Docket No.: 532212000623
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Thomas L. CANTOR

Application No.: 10/617,489 Confirmation No.: 4476

Filed: July 10, 2003 Art Unit: 1641

For: METHODS, KITS AND ANTIBODIES FOR DETECTING PARATHYROID HORMONE Examiner: J. Cheu

DECLARATION OF THOMAS L. CANTOR PURSUANT TO 37 C.F.R § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Thomas L. Cantor, in my individual capacity, hereby declare as follows:

1. I am the inventor of the above-referenced patent application, and am familiar with the contents thereof.

2. This application is a continuation-in-part (CIP) of serial number 09/344,639, filed on June 26, 1999, and now issued as U.S. Patent No. 6,743,590, which is a CIP of serial number 09/231,422 (the '422 application), filed on January 14, 1999, now issued as U.S. Patent No. 6,689,566 (the '566 patent).

3. Figure 5 of the '422 application mentions a tracer antibody that is referred to as "PTH 1-8 Antibody as Tracer." The term "PTH 1-8 antibody" was used to refer to this antibody

because I then believed the antibody had been isolated by Dr. Ping Gao and his co-workers using a PTH 1-8 peptide for affinity purification of the antibody. When Figure 5 was created and when the '422 application was filed, I believed that such a tracer antibody was used for the experiment represented by Figure 5.

4. In a deposition which occurred on August 27, 2003, related to the case Nichols v. Scantibodies on Nichols's U.S. patent No. 6,030,790, the attorney questioning me said, "Well, isn't it true that 1-9 meant that you used a 1-9 peptide to affinity purify your antibody?" In response, I stated: "I believe Dr. Gao used a peptide that contained 1-9."

5. In connection with the case Scantibodies v. Immutopics on Scantibodies' 566 patent, I became aware that the peptide in question was purchased from a supplier, as was the normal practice at Scantibodies: such peptides are typically purchased, rather than made. I also saw the invoice for the particular peptide used for affinity purification of the antibody that was used to generate the data represented by Figure 5 of the patent application serial number 09/231,422, which was filed on January 14, 1999. The invoice states that the peptide purchased for that purpose was a peptide containing PTH 1-9.

6. Based on the above facts, I believe that the antibody used to generate the results described in Figure 5 of serial number 09/231,422 should have been referred to as a PTH 1-9 antibody. In the current application, Figure 5, which was retained from the earlier '422 application, still refers to the antibody as "PTH 1-8 Antibody." Accordingly, I believe Figure 5 of the current application should be corrected to refer to the antibody as "PTH 1-9 Antibody."

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

March 6, 2006
Date

Thomas L. Cantor

REBUTTAL EXPERT REPORT OF RICHARD A. LERNER, M.D.

I, Richard A. Lerner, M.D., submit this rebuttal expert report on behalf of Scantibodies Clinical Laboratories, Inc. and Scantibodies Laboratories, Inc. (collectively "Scantibodies"). A copy of my *curriculum vitae* and list of publications was attached as Exhibit 1 to my February 2003 declaration in support of Scantibodies' motion for summary judgment pursuant to 35 U.S.C. § 102(b).

1. Compensation and Prior Testimony

A list of all materials provided to me by counsel for Scantibodies in this case is attached as Exhibit 1. For my total work related to this case (which began in October 2002 and which will run through trial), I am being compensated in the amount of $50,000. My compensation is not contingent upon the outcome of this matter.

2. Scope Of Opinions Rendered In This Report

I understand that Nichols Institute Diagnostics, Inc.'s ("Nichols") asserts that Scantibodies' Whole, CAP and DUO PTH assay kits infringe the claims of United States Patent No. 6,030,790 (the "'790 patent"). I further understand that Nichols has asserted all 25 claims of the '790 patent against Scantibodies' kits, and that Nichols asserts both literal infringement and infringement under the doctrine of equivalents. This report summarizes the opinions about which I may testify at the trial on behalf of Scantibodies regarding those allegations of infringement.

I have reviewed certain opinions issued by the Court in this case, as well as various pleadings, declarations and other testimony. In my opinion, none of Scantibodies' accused

devices infringe any of the 25 asserted claims, either literally or under the doctrine of equivalents. The bases for my opinions, which are set out more fully below, include:

a. My review of the '790 patent, as well as my review of the supplemental expert report submitted by Dr. Leonard Deftos, and the attachments to his report;

b. My nearly 35 years of research in the field of immunology, including my expertise in anti-peptide antibodies and how antibodies react with antigens;

c. My review and understanding of the Court's Order Construing Patent Claims And Terms For Jury Trials (dated March 10, 2003), the Court's Order Denying Defendants' Motion for Summary Judgment and Granting Summary Adjudication (dated June 2, 2003), other documents, deposition testimony and exhibits; and

d. Analysis performed in my laboratory under my direction on the antibodies used in the Scantibodies' assays, including the so-called "PTH(1-9)" antibody as well as the "PTH(1-12)" antibody.

If asked, I also may testify in rebuttal to the testimony, opinions and evidence presented by Nichols regarding both validity and infringement of the '790 patent. I also may provide a general overview of immunology, immunoassays, anti-peptide antibodies, and how antibodies and antigens interact.

3. My Background

I am the President of the Scripps Research Institute in La Jolla, California. The Scripps Research Institute is the country's largest, private, non-profit research organizations and stands

at the forefront of basic biomedical science, a vital segment of medical research that seeks to comprehend the most fundamental processes of life. The Institute has become internationally recognized for its basic research into immunology, molecular and cellular biology, chemistry, neurosciences, autoimmune diseases, cardiovascular diseases and synthetic vaccine development. Particularly significant is the Institute's study of the basic structure and design of biological molecules; in this arena TSRI is among a handful of the world's leading centers. Indeed, in each of 2001 and 2002, a member of TSRI's Chemistry Department has been awarded the Nobel Prize in Chemistry. Currently housed in fourteen laboratory buildings, the Institute's staff includes more than 270 professors, 800 postdoctoral fellows, 1500 laboratory technicians, administrative and support personnel, and 126 Ph.D. students.

I am competent to render the expert testimony set forth in this declaration based on my experience and education in the fields immunology and molecular biology. I attended Northwestern University and graduated from Stanford Medical School. I served as Chairman of the Department of Molecular Biology of the Institute from 1982-1986 prior to assuming the presidency of TSRI.

I have received numerous prizes and awards, including the Parke Davis Award in 1978, John A. Muntz Memorial Prize in 1990, San Marino Prize in 1990, The Burroughs Wellcome Fund and the FASEB, Wellcome Visiting Professor Award in 1990, The College De France Lectureship in 1991, Arthur C. Cope Scholar Award in 1991, The Tenth Annual Jeanette Piperno Memorial Award in 1991, Sixteenth Annual CIBA-GEIGY Drew Award in Biomedical Research in 1992, Humboldt Research Award in 1994, the Wolf Prize in Chemistry in 1994-1995, the California Scientist of the Year Award in 1996, the William B. Coley Award for Distinguished Research in Basic and Tumor Immunology, Cancer Research Institute, New

York; and Windaus-Medal/Award, Georg-August-Universitat Gottingen, Gottingen, Germany. In 2003 I received the Paul Ehrlich and Ludwig Darmstaedter Prize for achievements in connection with the development of catalytic antibodies. The award was given by the Scientific Council of the Paul Ehrlich Foundation in Frankfurt, Germany.

I also serve on the editorial boards of the following scientific journals: the Journal of Virology Molecular Biology and Medicine, Vaccine, In Vivo, Peptide Research, Bioorganic and Medicinal Chemistry Letters, Drug Targeting and Delivery, Senior Contributing Editor to PNAS and Chemistry and Biology, Bioorganic and Medicinal Chemistry and Molecular Medicine, and Catalysis Technology. I have been named to numerous boards and scientific academies throughout the world in my fields of expertise, including the Royal Swedish Academy of Sciences in 1985; The Neurosciences Research Foundation, Inc. in 1992; Member, Advisory Board, Chemical & Engineering News in 1994, Member, ETH Institute of Biotechnology Advisory Board, Zurich, in 1994; and California Council on Science and Technology Board of Directors, 1996-1997.

I am listed as an inventor or co-inventor on over 50 issued patents in the United States, most of which are in fields related to the issues in this case. While I have not specifically studied the PTH hormone, I have studied numerous other peptide hormones and am well-qualified to discuss how antibodies recognize and bind with hormones such as PTH. I have spent my professional life studying the immune system and the role played by antibodies in it. I discovered and pioneered the conversion of antibodies into enzymes. I have authored over 400 articles that have been published in academic journals related to these fields.

4. Summary of Infringement Analysis

I understand that a product literally infringes a patent claim if each and every claim element is present in the accused product. I further understand that an accused product infringes a claim under the doctrine of equivalents where, although each claim element is not present in the accused product, the product or device has substantially the same function, works in substantially the same way, accomplishes substantially the same result.

It is my opinion that none of the accused products literally infringe claims 1-25 of the '790 patent because each of the accused products is missing one or more of the limitations of these claims. It is also my opinion that none of the accused products infringe claims 1-25 of the '790 patent under the doctrine of equivalents.

5. Analysis Performed On Scantibodies' Tracer Antibodies

In preparation to render the opinions set forth in this report, I directed analysis (performed under my supervision in my laboratory at TSRI) of the antibodies used in the Scantibodies' assays, including the so-called "PTH(1-9)" antibody as well as the "PTH(1-12)" antibody. I designed the research protocol and reviewed the results of each experiment. The results of those experiments are attached as Exhibits 2 through 7 to this report. These tests were performed on July 21, 2003, July 23-4, 2003, July 28, 2003, September 18, 2003 and September 23, 2003.

In the experiments, a research associate in my laboratory performed titration analysis of two Scantibodies tracer antibodies, using direct ELISA's. It is my understanding that both antibodies were raised by injecting a goat with the whole PTH(1-84) molecule and then purifying

the antisera using one of two synthesized peptides: PTH(1-9) or PTH(1-12). The PTH(1-9) antibody is referred to in some results as the "N-terminal PTH" antibody. The tests dated July 21, 2003 (Exhibit 2) demonstrated that, while the PTH(1-9) had a high titer for the entire PTH molecule (1-84), it had a lower titer for the peptide 1-34, and none at all for the peptide 2-37.

In the direct ELISA's conducted July 23, 2003 (Exhibit 3), the PTH(1-9) antibody (shown in plates 3 and 4) had a high titer for the entire PTH molecule (1-84). The PTH(1-9) antibody also bound with the peptides 1-34 and 1-9, but had a much lower titer for those two peptides than to the entire PTH molecule. This was particularly true in plate 3.

In the direct ELISA's run on July 28, 2003 and on September 18, 2003 (Exhibits 5 and 6), we analyzed the PTH(1-9) antibody and the PTH(1-12) antibody, respectively. In each case, the antibody was analyzed against the entire PTH molecule (1-84), as well as numerous peptides: 1-5, 1-6, 1-7, 1-8 and 1-10 (corresponding to SEQ. ID. Nos. 1 and 3-6) of the '790 patent. The results from the titration analysis of the PTH(1-9) antibody show that it did not bind the peptides 1-5, 1-6, 1-7, 1-8 and 1-10. As discussed in the paragraph above, the results dated July 23, 2003 show that the PTH(1-9) antibody recognized the peptide 1-9. That antibody had a much lower titer for the peptide 1-9 (SEQ. ID. No. 2) and showed far better binding with the entire PTH molecule (1-84) than it did to the 1-9 peptide.

Likewise, the direct ELISA's dated September 18, 2003 on the PTH(1-12) antibody show that it did not bind with the peptides 1-5, 1-6, 1-7, 1-8 and 1-10 (SEQ. ID. Nos. 1 and 3-6). It did bind the peptide 1-9 (SEQ. ID. No. 2), but the titer was much lower than the results for the antibody binding with the entire PTH molecule. The PTH(1-12) antibody demonstrated a high titer to the whole PTH molecule (1-84).

In the final experiment dated September 23, 2003 (Exhibit 7), we performed a series of competition assays. In the first set (plate 1), we analyzed the PTH(1-9) antibody. We began with a known quantity of the entire PTH molecule (1-84). We then added various concentrations of two peptides (both lacking an "intact N-terminus"): 3-34 and 4-37. In the tests for the PTH(1-9) antibody, the peptide 3-34 demonstrated more competitive binding than the other peptide (4-37). In both cases, however, the degree of binding by the PTH(1-9) antibody to these N-terminally truncated (and "inactive") fragments was significant. The same was true for the analysis of the PTH(1-12) antibody, where the same protocol was followed. In the data from the PTH(1-12) antibody, however, the "best" competitor was the entire PTH molecule (1-84). As with the PTH(1-9) antibody, the degree of binding by the PTH(1-12) antibody to 3-34 and 4-37 (the N-terminally truncated and "inactive") fragments was significant.

In summary, the direct ELISA analysis of the PTH(1-9) antibody and the PTH(1-12) antibody shows that neither binds to the peptides of PTH 1-5, 1-6, 1-7, 1-8 and 1-10. While each of the two antibodies exhibits some binding with the 1-9 peptide of PTH, they have a much higher binding affinity for the entire PTH molecule (1-84) than with the 1-9 peptide. In my opinion, neither of the Scantibodies antibodies are selective for the 6 peptides having "SEQ. ID. Nos. 1-6" recited in the '790 patent. The antibodies do not "selectively" bind with peptides having "SEQ. ID. Nos. 1-6". In addition, the results of the competition assays show that the PTH(1-9) and PTH(1-12) antibodies have significant binding with fragments of the PTH molecule that lack the first two amino acids (serine and valine). It is my understanding that fragments of PTH without the first two amino acids are not biologically active, as Dr. Deftos states at paragraph 21 of his report ("Thus, in order for PTH to be biologically active, its N-terminal portion must be 'intact;' *e.g.,* at least the first amino acid or two must be present.").

Accordingly, the two Scantibodies tracer antibodies are not "selective" for "biologically active" peptides or fragments of PTH.

6. Opinions in Rebuttal to Dr. Deftos

a. The Tracer Antibody in Scantibodies' Kits Does Not "Selectively Bind" Peptides From Among PTH(1-10), PTH(1-9), PTH(1-8), PTH(1-7), PTH(1-6) and PTH(1-5), As Required By Claims 1-25.

All of the claims of the '790 patent claim an antibody that selectively binds to peptides having "SEQ. ID Nos. 1-6." That is, the antibodies *selectively bind* to *peptides* from among PTH(1-10), PTH(1-9), PTH(1-8), PTH(1-7), PTH(1-6) and PTH(1-5). In Its March 10, 2003 Order, the Court construed the phrase "selectively binds" to mean that an antibody "seeks out specifically and attaches to a specific arrangement of atoms or molecules". March 10, 2003 Order at 14.

There are two important, over-arching reasons underlying my opinion that Scantibodies' kits do not contain a tracer antibody that "selectively binds" to those specific peptides. First, the Court has defined the term "selectively binds" in a way that correctly emphasizes the essence of immunology: the specificity of antibodies in an antibody/antigen interaction. In his report, Dr. Deftos changes the Court's definition in a fundamental, and I believe, incorrect way. According to Dr. Deftos, "an antibody that would recognize and attach *preferentially* to bioactive PTH would selectively bind to biologically active PTH." Deftos Supplemental Report at ¶ 13. (Emphasis added). Dr. Deftos incorrectly equates "preferential" binding with "selective" binding. I disagree with Dr. Deftos. A high affinity antibody also could react with other antigens, such as other peptides of PTH. That same antibody, however, would not be said to be

"selective" for one antigen; the antibody is incapable of specifically discriminating between these antigens. When an antibody specifically binds to an antigen, it means that the antibody binds with selectivity that is sufficient to discriminate or differentiate that antigen from other substances in the sample.

My second fundamental disagreement with Dr. Deftos is related to antibody "selectivity." The patent claims antibodies that bind specifically to six particular peptides, not epitopes on the entire 84 amino acid PTH molecule. The patent claims antibodies that will select out and bind specific peptides having "SEQ. ID Nos. 1-6." The patent clearly defines "SEQ. ID Nos. 1-6" in the patent specification. "SEQ. ID Nos. 1-6" means the following peptides: PTH(1-10), PTH(1-9), PTH(1-8), PTH(1-7), PTH(1-6) and PTH(1-5).

The phrase "peptides having SEQ. ID Nos. 1-6" does not refer to regions or epitopes within the entire PTH molecule. It refers to these six specific peptides for at least two reasons. First, the patent specification defines them as peptides, not as epitopes within PTH(1-84). Second, the other claims of the patent (see Claim 1 for example) clearly draw a distinction between "a peptide of hPTH selected from the group consisting of peptides having SEQ. ID Nos. 1-6" and "an epitope" along the PTH molecule (e.g., between amino acids 24-37). In addition, the definitions of "SEQ. ID Nos. 1-6" found at the end of column 2 set forth peptides (SEQ. ID No. 6, for example) made up of the first 5 amino acids found in the PTH(1-84) molecule, as well as an "amino group" (NH_2) and a "carboxyl group" (OH). Thus, an antibody that selectively binds to SEQ. ID. No. 6 means an antibody that selectively binds to that free-standing 5-amino acid peptide indicated by the amino and carboxyl groups. It does not mean the epitope on the 84-amino acid PTH molecule between amino acids 1-5. The "epitope" between amino acids 1-5 on the entire PTH(1-84) molecule would not be indicated by both the amino and carboxyl groups

that are found in the structure of the synthesized peptide having SEQ. ID. No. 6. The Court emphasized this point by defining "epitope" to mean "a specific arrangement of amino acids located on a peptide or protein to which an antibody or antibody fragment binds." March 10, 2003 Order at 14.

In any event, none of the accused Scantibodies devices contain antibodies that "selectively bind" to peptides having "SEQ. ID Nos. 1-6", as those SEQ. ID. Nos. are defined in the specification of the '790 patent. The Court has defined "selectivity" to mean "specificity." ["Selectively binds" means that an antibody "seeks out specifically and attaches to a specific arrangement of atoms or molecules". March 10, 2003 Order at 14]. According to the Court's definition, the claimed antibody recognizes and reacts with the peptide of interest (e.g., PTH(1-10), PTH(1-9), PTH(1-8), PTH(1-7), PTH(1-6) or PTH(1-5)), discriminating it from other substances present in the sample. That is, the antibodies will not selectively or specifically bind PTH(1-10), PTH(1-9), PTH(1-8), PTH(1-7), PTH(1-6) or PTH(1-5). In the context of the direct ELISA's performed in my laboratory demonstrate, Scantibodies' PTH(1-9) antibody and PTH(1-12) antibody are not selective for the shorter peptides claimed (having SEQ. ID. Nos. 1-6) in the '790 patent.

For these reasons, it is my opinion that none of the accused Scantibodies products or services literally infringe claims 1-25 of the '790 patent, which all require an antibody that "selectively binds" at least one of the peptides having "SEQ. ID Nos. 1-6". It is also my opinion that none of the accused products or services infringe any of the 25 claims under the doctrine of equivalents. An assay containing a tracer antibody that will selectively bind whole PTH (that is, 1-84) is substantially different than an assay containing a tracer antibody that will selectively

bind these six, short peptides recited in the patent claims (namely, those peptides having "SEQ. ID Nos. 1-6").

b. The Tracer Antibody In Scantibodies' Kits Binds Inactive Fragments of PTH, Or Fragments of PTH Lacking The First Two Amino Acids

Selectivity, or specificity, is at the heart of what Nichols claims is the alleged novel feature of the antibody claimed in the '790 patent's claims. ("The allegedly unique feature of the '790 patent is that it claims a description of antibodies that selectively bind *only* to peptides with the N-terminus intact.") (June 2, 2003 Order at 4). Under the Court's claim construction, the Scantibodies devices do not infringe the asserted claims because they do not satisfy the "selectivity" requirements of the 25 asserted claims.

The two Scantibodies tracer antibodies are not "specific" or "selective" for the peptides claimed in the '790 patent. The Scantibodies antibodies cross-react with peptides that *do not* contain an intact N-terminus (e.g., those peptides of PTH lacking the first two amino acids). According to Dr. Deftos' own analyses, the tracer antibody used in the Scantibodies' assays binds with PTH(3-34) when present in competition experiments with PTH(1-84). Dr. Deftos' own analysis establishes that the PTH(3-34) peptide exhibits 11% inhibition with sandwich formation. (Deftos Supplemental Report at ¶ 84 and Ex. 26). An antibody with that degree of binding to inactive PTH cannot be said to be "specific" or "selective" for "biologically active" PTH or with the peptides PTH(1-10), PTH(1-9), PTH(1-8), PTH(1-7), PTH(1-6) or PTH(1-5).

Dr. Deftos' results are confirmed by the data from my laboratory's competition assays for both the PTH(1-9) and PTH(1-12) antibodies. In the data from experiments dated September 23, 2003 (Exhibit 7), the degree of binding by the PTH(1-9) antibody and the PTH(1-12) antibody to

two N-terminally truncated, "inactive" fragments (3-34 and 4-37) was significant. Under the Court's claim construction as explained in the June 2, 2003 Order, the Scantibodies devices do not literally infringe any of the asserted claims 1-25. Neither antibody satisfies the "selectivity" requirements of the 25 asserted claims. It is also my opinion that none of the accused products or services infringe any of the 25 claims under the doctrine of equivalents. An assay that will bind with inactive fragments of PTH such as 3-34 and 4-37 (those lacking the first two amino acids) is substantially different than an assay that will bind solely biologically active PTH, or N-terminally "intact" PTH fragments.

c. The Scantibodies' Kits Do Not Contain a Capture Antibody That Selectively Binds hPTH At An Epitope Contained Within Amino Acids 24-37, As Required By Claims 1-16 and 18-19.

Dr. Deftos acknowledges that Scantibodies' witnesses testified at deposition that the capture antibody used in all Scantibodies' PTH products binds the PTH(1-84) molecule between amino acids 39-84. Despite that fact, Dr. Deftos opines that Scantibodies' capture antibody binds PTH(1-84) between amino acids 24-37. Unlike his analysis of the tracer antibody, Dr. Deftos does not rely on any scientific analysis examining where the Scantibodies capture antibody binds the PTH molecule. The only "evidence" Dr. Deftos points to in support of his opinion is a conceptual diagram in a Scantibodies brochure depicting a sandwich assay being formed in relation to a PTH molecule. *See* Deftos Supplemental Report at ¶ 53. The brochure does not make any statement as to where the Scantibodies capture antibody binds PTH(1-84). Because the capture antibody binds PTH at an epitope between amino acids 39 and 84, it is my opinion that Scantibodies' kits do not literally infringe claims 1-16 or claims 18-19 of the '790 patent.

It is also my opinion that Scantibodies' kits do not infringe claims 1-16 or claims 18-19 under the doctrine of equivalents. In the PTH assay using a capture antibody at the epitope between amino acids 24-37 (such as that described in claims 1-16 of the '790 patent), the assay will capture whole PTH (that is, PTH (1-84)), but will also capture additional fragments, such as PTH(1-37). In an assay using a capture antibody binding at an epitope between amino acids 39-84 (such as that used in Scantibodies' kits), the whole PTH molecule (PTH (1-84)) will be captured. Fragments such as PTH(1-37), for example, will not be captured. PTH assays employing two different capture antibodies (one at the epitope between amino acids 24-37 and the other at an epitope between amino acids 39-84) have substantial differences, in my opinion, especially where the patent claims an assay for detecting PTH(1-37) or PTH(1-34). (*See* '790 patent at col. 1:6-31).

d. The Scantibodies' Kits Do Not Contain a "Suitable Carrier" As Required By Claims 17-25.

Scantibodies' assays also do not infringe claims 17-25 as they do not contain the "suitable carrier" required by those claims (as the Court has construed that term). According to the Court, a "suitable carrier" is "[a]ny substance that serves to facilitate the ability of an antibody to seize an antigen. Since antibodies and antigens vary greatly, a suitable carrier would be one or more substances which maximize the immunoassay process for the particular antibodies and antigens sought. A suitable carrier may be a liquid or a solid." March 10, 2003 Order at 14. In his report, Dr. Deftos states (in one brief sentence) that this claim element is present in Scantibodies' assays. According to Dr. Deftos, "Scantibodies' Whole, CAP and DUO PTH assay kits each contain a composition comprising an N-terminal antibody having the

claimed characteristics (binding the N-terminus of biologically active PTH) and at least one suitable carrier such as sodium azide and buffer." Deftos Supplemental Report at ¶ 60.

Dr. Deftos' report lacks any analysis as to why sodium azide or the buffer "serves to facilitate the ability of an antibody to seize an antigen." Dr. Deftos appears to merely conclude, without any analysis of these substances' function, that they serve "to facilitate the ability of an antibody to seize an antigen." Substances such as sodium azide or "buffers" are added to immunoassays in order to preserve the antibodies during the shelf life of the assay. That is, the substances generally are added to preserve the antibodies. For example, they are added to prevent bacteria from contaminating the antibodies in the kit. They are not added to "facilitate" the ability of an antibody "to seize" an antigen. Moreover, the Court also has defined "suitable carrier" to include "one or more substances which maximize the immunoassay process for the particular antibodies and antigens sought." March 10, 2003 Order at 14. This suggests that the "carrier" is chosen in a particular assay to enhance the binding of antibodies for one antigen (namely, hPTH or a peptide of hPTH). Again, there is nothing in Dr. Deftos' report to support the conclusion that sodium azide or other buffers were chosen or added to the Scantibodies kit because those carriers "maximize the immunoassay process" for PTH or peptides if PTH.

It is my opinion that Scantibodies' assays do not employ a composition with a "suitable carrier" required in claims 17-25, as the Court has defined that term. Therefore, the accused Scantibodies' devices do not infringe claims 17-25, either literally or under the doctrine of equivalents.

7. Conclusion

For at least the reasons stated above, I do not believe any of the claims of the '790 patent is infringed by any of the accused Scantibodies PTH products or services. I may testify about any of the preceding topics at trial. I reserve the right to respond to any reports that are submitted by plaintiffs' expert witnesses or to any testimony by plaintiffs' fact or expert witnesses, whether at deposition or trial.

Dated: December 2, 2004

Richard A. Lerner, M.D.

EXHIBIT 1

Documents Sent to R. Lerner

Date Sent	Document	Bates Range
10/17/2002	U.S. Patent No. 6,030,790	
11/5/2002	"A New Immunoenzymometric Assay for BioActive N-Terminal Human Parathyroid Hormone Fragments and Its Application in Pharmacokinetic Studies in Dogs"	
	"Production of Sequence Specific Polyclonal Antibodies to Human Parathyroid Hormone 1-37 by Immunization with Multiple Antigenic Peptides"	
11/21/2002	Biosketch: L.J. Deftos	
	Information re: Joseph Oliver Falkingham, III	
11/27/2002	"P142 Immunological Detection of Human Parathyroid Hormone 1-37 (hPTH 1-37), The Physiologically Circulating Fragment of hPTH"	
	"A New Immunoenzymometric Assay for BioActive N-Terminal Human Parathyroid Hormone Fragments and Its Application in Pharmacokinetic Studies in Dogs"	
	"Production of Sequence Specific Polyclonal Antibodies to Human Parathyroid Hormone 1-37 by Immunization with Multiple Antigenic Peptides"	
1/24/2003	Declaration of Dr. Leonard J. Deftos in support of Plaintiff Nichols Institute Diagnostics, Inc.'s Second Supplemental Claim Construction Brief (w/exhibits)	
2/6/2003	"P142 Immunological Detection of Human Parathyroid Hormone 1-37 (hPTH 1-37), The Physiologically Circulating Fragment of hPTH"	
12/1/2004	Peptide experiments from 7/21/03 - 9/23/03	RL 0001 - RL 0017

EXHIBIT 2

ELISA: Peptides PTH. Date: 7.21.03

Conjugates: 1-34 / 1-84 / 2-37 (Peptides) Dilutions: 1 mg/ml → 0.1 ug/well in PBS

Blocking Time:	15'	2° Ab Incubation Time:	30'	Rabbit x Goat 1:1000
1° Ab Incubation Time:	1hr	Developing Time:	30'	

1-34: Bachem H-4835 lot# 0654976
1-84: Bachem H-1370.1000 lot# 804356
2-37: Abgent 5306/304-1 lot# SD30418b

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A	Gt x H PTH (1-84) Lot # 0990-012792 Cat# IAG0229											
B	Gt x H N-terminal PTH Lot # F518D Cat# 3AG1128											
C	Gt x H PTH (1-84)											
D	Gt x H N-terminal PTH											
E	Gt x H PTH (1-84)											
F	Gt x H N-terminal PTH											
G												
H												

1-34 (A, B) 1-84 (C, D) 2-37 (E, F) — 1.09 mg/ml

All at 1:110 dilution

All antibodies from Scantibodies

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Optical Density (raw)

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.191	0.140	0.119	0.138	0.129	0.142	0.139	0.137	0.136	0.156	0.225	0.248
B	1.422	0.382	0.207	0.222	0.146	0.144	0.171	0.119	0.144	0.205	0.209	0.359
C	2.034	1.729	1.342	1.048	0.637	0.529	0.304	0.249	0.285	0.364	0.248	0.437
D	2.192	1.713	1.154	0.956	0.264	0.223	0.224	0.175	0.180	0.251	0.242	0.236
E	0.162	0.129	0.140	0.145	0.125	0.127	0.126	0.140	0.146	0.248	0.187	0.175
F	0.138	0.130	0.118	0.133	0.142	0.119	0.133	0.165	0.237	0.206	0.184	0.166
G	0.078	0.086	0.081	0.078	0.075	0.071	0.075	0.077	0.078	0.077	0.076	0.080
H	0.074	0.076	0.078	0.076	0.078	0.078	0.077	0.076	0.081	0.082	0.078	0.077

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
2:25 PM 7/21/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

PEPTIDE

EXHIBIT 3

ELISA: Peptide Titering Date: 7.23.03

Peptides 1-9, 1-34, 1-84

~~Conjugates~~: 2-9, 2-37, 37-82 (4°C ON)

Dilutions: 1mg/ml → 1/250 in PBS or 50mM Borate Buffer pH 9.0

Blocking Time:	30'	2° Ab Incubation Time:	1hr
1° Ab Incubation Time:	1hr	Developing Time:	30'

@ 0.1ug/well

Ag

Plate# 1

	1	2	3	4	5	6	7	8	9	10	11	12
1-9	A	–									–	
1-34	B											
1-84	C	GαH pTH (1-84) plasma (1:10)									8	
2-9	D										64+	
2-37	E										–	
~~37-82~~	F										–	
PBS	G											
	H											

Plate# 2

	1	2	3	4	5	6	7	8	9	10	11	12
1-9	A										–	
1-34	B									–	16	
1-84	C	GαH pTH (1-84) plasma (1:10)									8	
2-9	D										–	
2-37	E										–	
37-82	F										16+	
50mM Borate pH 9.0	G											
	H											

Plate# 3

	1	2	3	4	5	6	7	8	9	10	11	12
1-9	A										8+	
1-34	B										16+	
1-84	C	GαH N-terminal pTH (1:10)									64+	
2-9	D										–	
2-37	E										–	
~~37-82~~	F											
PBS	G											
	H											

ELISA: _____ **Date:** _____

Conjugates: _____ **Dilutions:** _____

Blocking Time:		*2 ° Ab Incubation Time:*	
1 ° Ab Incubation Time:		*Developing Time:*	

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A											8+	
B											8+	
C		GLH	N-terminal		PTH			(1-10)			—	
D											—	
E											—	
F											—	
G												
H												

1-9
1-34
1-84
2-9
2-37
37-82
10mM Borate pH 9.0

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Optical Density (raw)

2 4 8 16 32 64 128 256 Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.295	0.313	0.151	0.125	0.113	0.112	0.143	0.122	0.146	0.148	0.154	0.294
B	3.070	2.964	1.519	0.257	0.167	0.139	0.145	0.126	0.150	0.186	0.169	0.413
C	3.938	3.895	3.764	3.812	3.276	2.566	1.766	1.187	0.909	0.473	0.303	0.493
D	0.323	0.182	0.149	0.131	0.125	0.123	0.135	0.145	0.142	0.149	0.178	0.167
E	0.432	0.181	0.150	0.164	0.130	0.120	0.154	0.181	0.139	0.144	0.164	0.157
F	0.390	0.204	0.161	0.128	0.140	0.120	0.129	0.203	0.136	0.168	0.158	0.177
G	0.110	0.112	0.111	0.120	0.142	0.118	0.122	0.153	0.175	0.174	0.221	0.177
H	0.098	0.112	0.116	0.140	0.144	0.138	0.157	0.159	0.220	0.253	0.260	0.471

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
1:15 PM 7/23/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

PEP 1

Optical Density (raw)

2 4 8 16 32 Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.152	0.410	0.262	0.217	0.149	0.146	0.155	0.161	0.145	0.147	0.143	0.184
B	1.896	1.107	0.832	0.927	0.852	0.629	0.667	0.568	0.520	0.581	0.810	0.857
C	0.989	0.733	0.609	0.368	0.241	0.225	0.167	0.154	0.160	0.143	0.141	0.184
D	0.390	0.268	0.222	0.147	0.155	0.156	0.148	0.141	0.116	0.110	0.135	0.121
E	0.311	0.319	0.208	0.201	0.134	0.150	0.125	0.125	0.111	0.106	0.170	0.117
F	3.998	3.832	3.670	2.352	1.694	1.036	0.835	0.411	0.281	0.188	0.175	0.126
G	0.206	0.269	0.200	0.200	0.114	0.125	0.124	0.150	0.103	0.120	0.118	0.117
H	0.316	0.256	0.142	0.083	0.133	0.185	0.128	0.121	0.119	0.109	0.139	0.117

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
1:17 PM 7/23/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

PEP 2

Optical Density (raw)

2 4 8 16 32 64 128

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.425	3.801	3.654	0.707	0.241	0.194	0.189	0.146	0.149	0.143	0.223	0.124
B	4.000	3.722	3.497	2.395	0.955	0.293	0.222	0.164	0.155	0.136	0.084	0.351
C	4.000	3.784	3.896	4.000	3.798	2.980	1.164	0.508	0.252	0.200	0.156	0.165
D	0.267	0.331	0.706	0.216	0.191	0.193	0.154	0.148	0.137	0.125	0.138	0.134
E	0.353	0.235	0.183	0.244	0.184	0.184	0.139	0.129	0.088	0.125	0.110	0.131
F	0.912	0.436	0.248	0.186	0.272	0.200	0.161	0.148	0.177	0.171	0.130	0.152
G	0.468	0.342	0.445	0.235	0.193	0.244	0.136	0.162	0.156	0.118	0.126	0.120
H	0.480	0.420	0.284	0.174	0.224	0.144	0.214	0.120	0.130	0.165	0.129	0.119

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
1:20 PM 7/23/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

PEP 3

Optical Density (raw)

2 4 8 16 32 64 128 Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.836	4.000	4.000	0.405	0.195	0.162	0.174	0.172	0.174	0.139	0.133	0.164
B	4.000	4.000	3.695	1.685	1.049	0.631	0.801	0.674	0.647	0.825	0.520	1.420
C	0.444	0.278	0.292	0.204	0.184	0.143	0.187	0.118	0.136	0.131	0.129	0.129
D	0.207	0.185	0.259	0.207	0.192	0.154	0.140	0.133	0.122	0.130	0.113	0.115
E	0.183	0.199	0.363	0.173	0.173	0.167	0.129	0.122	0.115	0.132	0.117	0.124
F	0.181	0.172	0.177	0.179	0.177	0.185	0.153	0.124	0.122	0.140	0.115	0.122
G	0.248	0.289	0.210	0.237	0.170	0.155	0.191	0.140	0.156	0.137	0.133	0.118
H	0.188	0.202	0.437	0.224	0.158	0.195	0.172	0.167	0.098	0.165	0.126	0.145

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
1:21 PM 7/23/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

PEP 4

EXHIBIT 4

ELISA: Peptide Date: 7.24.03

Peptide Conjugates: 1-84, 5-12 (0.1 ug/well) Dilutions: 1mg/ml → 1/250 in PBS

Blocking Time:	15'	2° Ab Incubation Time:	30'
1° Ab Incubation Time:	1hr	Developing Time:	30'

Ag

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A	1-84	Gat	pTH	(1-84)	Lot #			0998-012		792		
B	5-12											
C	1-84	Gat	N-terminal	pTH	Lot #			F518.D				
D	5-12											
E												
F												
G												
H												

both ab @ 1:10

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Optical Density (raw)

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.388	3.211	3.167	3.056	2.667	2.460	1.932	1.607	1.199	0.815	0.538	0.375
B	0.512	0.280	0.424	0.175	0.129	0.117	0.116	0.104	0.102	0.094	0.094	0.106
C	2.673	2.633	2.635	2.498	2.403	2.324	1.889	0.876	0.352	0.175	0.134	0.120
D	0.910	0.091	0.128	0.137	0.090	0.090	0.096	0.103	0.083	0.092	0.100	0.090
E	0.047	0.052	0.048	0.048	0.047	0.048	0.048	0.047	0.047	0.047	0.047	0.046
F	0.047	0.047	0.047	0.047	0.048	0.048	0.049	0.053	0.054	0.047	0.048	0.046
G	0.046	0.047	0.048	0.047	0.049	0.046	0.047	0.051	0.050	0.047	0.045	0.046
H	0.049	0.048	0.049	0.047	0.048	0.046	0.047	0.046	0.046	0.047	0.047	0.047

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
1:56 PM 7/24/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

PEP

EXHIBIT 5

ELISA: Peptides Date: 7.28.03

Peptides: PTH: 1-5, 1-6, 1-7 } Synthetic Biomolecules
Conjugates: 1-8, 1-10, 1-84 } Bachem

Dilutions: 1mg/ml → 1/250 (0.1ug/well)

Blocking Time:	15'	2° Ab Incubation Time:	30'	G&R HRP 1:1,000
1° Ab Incubation Time:	1hr	Developing Time:	1hr	

Ab: G&H N-terminal PTH Cat# 3AG668 Lot #F518D Scantibodies

Ag:
1-5
1-6
1-7
1-8
1-10
1-84

Plate# 1 start @ 1:10 dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A	—											
B	—											
C	—											
D	—											
E	—											
F	64											
G												
H												

Ag?
Pep# 1200326
1200326
1200326
1200326
1200326
#-1370.100

Plate# 2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Optical Density (raw)

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.123	0.102	0.098	0.082	0.085	0.087	0.084	0.078	0.080	0.071	0.080	0.080
B	0.134	0.095	0.092	0.083	0.090	0.084	0.083	0.078	0.078	0.072	0.077	0.079
C	0.199	0.091	0.095	0.083	0.086	0.085	0.079	0.075	0.075	0.073	0.078	0.083
D	0.116	0.093	0.101	0.090	0.088	0.100	0.099	0.076	0.075	0.078	0.091	0.078
E	0.120	0.109	0.107	0.094	0.090	0.086	0.081	0.077	0.074	0.070	0.078	0.080
F	1.496	1.387	1.251	1.134	0.999	0.738	0.425	0.188	0.135	0.094	0.087	0.084
G	0.140	0.126	0.115	0.102	0.110	0.099	0.097	0.091	0.092	0.087	0.099	0.099
H	0.049	0.048	0.047	0.047	0.046	0.046	0.047	0.047	0.046	0.048	0.049	0.050

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
2:20 PM 7/28/03

Wavelength Combination: lLm1
Data Mode: Absorbance

PEPTIDE 1

EXHIBIT 6

ELISA: Ab 1-12 titering Date: 9.18.03

Peptides 1-5, 1-6, 1-7, 1-8, 1-9

~~Conjugates~~: 1-10, 1-84 Dilutions: 1mg/ml → dilute to 4ug/ml (PBS) + add 25 ul/plate = 0.1ug/well

Blocking Time:	30'	2° Ab Incubation Time:	30'
1° Ab Incubation Time:	1hr	Developing Time:	1hr

Ab 1-12 (Scantibodies)

ag

1-5
1-6
1-7
1-8
1-9
1-10
1-84

Plate# 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Ab	Starting	Concentration	@	1:10				—			
B									—			
C									—			
D									—			
E									1:16			
F									—			
G									1:256			
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Optical Density (raw)

Plate#1

1	2	3	4	5	6	7	8	9	10	11	12
0.226	0.183	0.201	0.144	0.248	0.166	0.188	0.219	0.131	0.191	0.279	0.224
0.175	0.181	0.119	0.155	0.155	0.178	0.169	0.188	0.142	0.227	0.225	0.203
0.140	0.182	0.131	0.133	0.166	0.151	0.142	0.137	0.167	0.250	0.147	0.432
0.142	0.137	0.150	0.200	0.136	0.191	0.172	0.170	0.126	0.172	0.196	0.185
3.872	3.666	2.818	1.895	1.135	0.679	0.453	0.260	0.227	0.192	0.162	0.202
0.333	0.362	0.173	0.153	0.138	0.187	0.167	0.189	0.188	0.162	0.149	0.161
3.745	3.723	3.966	3.626	3.658	3.578	2.261	1.157	0.576	0.368	0.221	0.390
0.330	0.121	0.133	0.136	0.141	0.142	0.139	0.161	0.227	0.392	0.411	1.083

Endpoint
Lm1 405
Automix: Once Calibrate: On

Plate Last Read:
2:59 PM 9/18/03

Wavelength Combination: lLm1
Data Mode: Absorbance

1-12

EXHIBIT 7

ELISA: Competition Date: 9.23.03

Peptide Conjugates: 1-84 Dilutions: 1mg/ml → 1/100,000

Competitors: 3-34, 4-37, 1-84 (pTH) Starting Concentration a 500um, serially diluted in 1/2.

Blocking Time:	30'	2° Ab Incubation Time:	1hr
1° Ab Incubation Time:	1hr	Developing Time:	30'

Ab: Gα4 N-terminal pTH # 3AG668 lot F518D

Plate# 1 1:200

	1	2	3	4	5	6	7	8	9	10	11	12
A (3-34)	500uM	250	125	62.5	31.25	15.625	7.8125	3.90625	1.9531			
B	0.9766	0.48828	0.24414	0.12207	0.06103	0.03051	0.01525	0.00762	0.00381			
C (4-37)											15nm	
D											122nm	
E (1-84)												
F											244nm	
G												
H												

Ab: Gα pTH (1-12) H3AG666 lot F588C (1.02 mg/ml)

Plate# 2

	1	2	3	4	5	6	7	8	9	10	11	12
A (3-34)												
B											1254M	
C (4-37)												
D											31uM	
E (1-84)												
F											244uM	
G												
H												

Plate#

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Optical Density (raw)

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.215	0.222	0.204	0.251	0.283	0.247	0.294	0.263	0.259	0.303	0.050	1.460
B	0.298	0.538	0.274	0.275	0.296	0.401	0.524	1.769	1.789	1.524	1.445	1.339
C	0.152	0.267	0.190	0.174	0.205	0.245	0.247	0.275	0.287	1.104	1.489	1.426
D	0.225	0.250	0.293	0.928	1.815	1.321	1.627	1.450	1.475	1.508	1.327	1.456
E	0.088	0.081	0.090	0.083	0.095	0.098	0.122	0.184	0.496	1.362	1.690	1.413
F	0.112	0.120	0.593	1.815	1.974	1.887	1.721	1.584	1.351	1.430	1.381	1.429
G	0.090	0.088	0.083	0.080	0.083	0.080	0.081	0.082	0.084	0.085	0.080	0.082
H	0.092	0.095	0.087	0.090	0.088	0.091	0.089	0.091	0.095	0.087	0.090	0.108

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
2:38 PM 9/23/03

Wavelength Combination: lLm1
Data Mode: Absorbance

NTERM COMP

Optical Density (raw)

Plate#1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.530	0.455	0.579	0.920	1.093	1.155	1.232	1.379	1.131	1.288	1.571	1.400
B	1.066	1.055	1.308	1.560	1.616	1.407	1.760	2.003	1.946	1.883	1.782	1.854
C	0.323	0.438	0.520	0.631	0.782	1.162	1.122	1.160	1.103	1.483	1.760	1.888
D	0.904	1.118	1.203	1.922	2.034	2.006	1.860	1.865	2.005	1.840	1.750	1.656
E	0.221	0.184	0.166	0.181	0.172	0.172	0.186	0.230	0.623	1.468	1.917	1.503
F	0.226	0.218	0.582	2.498	2.295	2.262	2.232	1.857	1.770	1.732	1.684	1.509
G	0.209	0.201	0.187	0.200	0.195	0.176	0.181	0.181	0.179	0.173	0.179	0.181
H	0.204	0.223	0.207	0.211	0.207	0.199	0.192	0.191	0.191	0.190	0.188	0.183

Endpoint

Lm1 405

Automix: Once
Calibrate: On

Plate Last Read:
2:39 PM 9/23/03

Wavelength Combination: !Lm1
Data Mode: Absorbance

1-12 COMP

Second Edition

CLINICAL IMMUNOLOGY

PRINCIPLES AND PRACTICE

Edited by

Robert R Rich

Thomas A Fleisher

William T Shearer

Brian L Kotzin

Harry W Schroeder Jr

Volume One

Mosby

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discrimination would imply infinite intrinsic affinity (negative free energy change of complex formation), which is not physically plausible.[17] Second, the convexity of atoms prevents perfect shape complementarity between antibody and antigen.[18]

In contrast to the epitope-centered forms of specificity just described, a third aspect of specificity relates to the ability of an antibody to discriminate between antigens that display many copies of one or more distinct epitopes. An antigen expressing many copies of one epitope is termed *multivalent*, and an antigen that expresses two or more different epitopes is referred to as *multideterminant* (Table 14.1). Because two different organisms may both express multiple copies of the same or a similar epitope, an antibody that is highly specific (in the first sense above) for such a shared epitope may be a poor discriminator between such multivalent particles.[19] Yet an antibody with a relatively poor degree of complementarity and intrinsic affinity for a given epitope, found on only one of two or more multivalent targets, may be superior at discriminating between these antigens. Furthermore, antibodies (or other molecules) expressing two or more binding sites with identical structures may not discriminate identically among antigens displaying the same epitope in different two- or three-dimensional distributions.[11]

Some final points regarding specificity: first, for many purposes, immunological specificity has an ultimately biological, not a physical, definition. If the end-point of analysis is the triggering of a complex response, such as cell activation or initiation of the complement cascade, then the presence, absence or extent of that response, and not the extent of receptor–antigen interaction, will be the ultimate criterion for evaluating specificity. Second, the enormous utility of antibodies is crucially dependent on the discriminatory abilities of these molecules with respect to other molecules or molecular aggregates. However, given that the discrimination mediated by antibodies is not absolute, the usefulness of a particular antibody may depend on which antigens or potential antigens are available for binding to the antibody. Third, apparent antibody specificity may vary with the methods used for analysis, as these methods may differ in sensitivity and in environmental conditions (pH, ionic strength, temperature).[20]

Protein epitopes

Several categories of epitope have been defined for protein antigens, based on the proximity of the relevant amino acids in the primary structure of the protein (Fig. 14.3). The simplest case is the *linear* epitope, where all of the amino acids constituting the epitope are derived from a contiguous stretch of the polypeptide chain. However, many – perhaps most – epitopes on globular proteins involve amino acids from two or more stretches of polypeptide that are distant from one another in the primary structure. Such an epitope is referred to as *conformational* or *discontinuous*. In some cases it is conceivable that a conformational epitope can comprise amino acids derived from separate, adjacent polypeptide chains. Another category of protein epitope, the *neo-epitope*, is reserved for those antigenic sites that become recognizable only after a post-translational event, such as proteolytic cleavage. For example, several neo-epitopes have been defined on cleavage products of human C1q, C3 and C9, components of the complement pathway.[21] Antibodies recognizing such neo-epitopes can be used to monitor the extent of activation of the complement pathway.[21]

The first structure of an antibody-variable module in complex with a globular protein antigen, determined by X-ray crystallography,[1] indicated that protein epitopes, defined on the basis of intermolecular contact, could be as large as 15–20 amino acids. A similar number of amino acids in the antibody V domains constituted the paratope. Of course, it is possible that there are smaller epitopes on globular proteins, particularly for regions of proteins that protrude or have a high radius of curvature. A recent crystallographic study suggests that a peptide antigen–antibody interaction can involve as many as 12 peptide amino acids in contact with the antibody.[22]

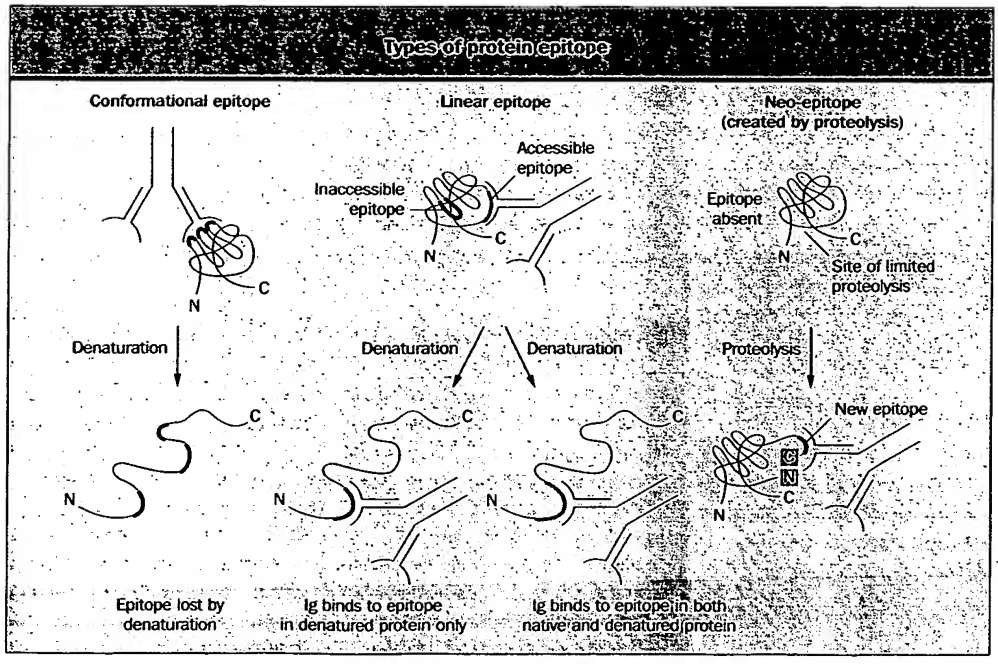

Figure 14.3 Types of protein epitope. Some antibodies recognize structural features of proteins that arise from the folding of the polypeptide backbone (conformational epitope). Other antibodies recognize groups of amino acid residues that are contiguous, or nearly so, in the primary (covalent) structure of the protein (linear epitope). If such a linear determinant is inaccessible in the native structure of the protein, the corresponding antibodies may only be elicited by the denatured form of the protein. Neo-epitopes are created by covalent post-translational modifications, such as proteolytic cleavage. (From Abbas AK, Lichtman AH, Pober JS. *Cellular and molecular immunology*, 3rd edn. WB Saunders 1997, with permission.)

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Structure of non-(1-84) PTH fragments secreted by parathyroid glands in primary and secondary hyperparathyroidism

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Structure of non-(1-84) PTH fragments secreted by parathyroid glands in primary and secondary hyperparathyroidism.

Background. Non-(1-84) parathyroid hormone (PTH) fragments are large circulating carboxyl-terminal (C) fragments with a partially preserved amino-terminal (N) structure. hPTH (7-84), a synthetic surrogate, has been demonstrated to exert biologic effects in vivo and in vitro which are opposite to those of hPTH (1-34) on the PTH/PTHrP type I receptor through a C-PTH receptor. We wanted to determine the N structure of non-(1-84) PTH fragments.

Methods. Parathyroid cells isolated from glands obtained at surgery from three patients with primary hyperparathyroidism and three patients with secondary hyperparathyroidism were incubated with ^{35}S-methionine to internally label their secretion products. Incubations were performed for 8 hours at the patient-ionized calcium concentration and in the presence of various protease inhibitors. The supernatant was fractionated by high-performance liquid chromatography (HPLC) and fractions were analyzed with PTH assays having (1 to 4) and (12 to 23) epitopes, respectively. The serum of each patient was similarly analyzed. Peaks of immunoreactivity identified were submitted to sequence analysis to recover the ^{35}S-methionine residues in positions 8 and 18.

Results. Three regions of interest were identified with PTH assays. They corresponded to non-(1-84) PTH fragments (further divided in regions 3 and 4), a peak of N-PTH migrating in front of hPTH (1-84) (region 2) and a peak of immunoreactivity corresponding to the elution position of hPTH (1-84) (region 1). The last corresponded to a single sequence starting at position 1. Region 2 gave similar results in all cases (a major signal starting at position 1) but also sometimes minor sequences starting at position 4 or 7. Regions 3 and 4 always identified a major sequence starting at positions 7 and minor sequences starting at positions 8, 10, and 15. Surprisingly, a major signal starting at position 1 was also present in region 3. The HPLC profile obtained from a given patient's parathyroid cells was qualitatively similar to the one obtained with his/her serum in each case.

Conclusion. These results indicate that non-(1-84) PTH fragments are composed of a family of fragments which may be generated by specific or progressive cleavage at the N region. The longest fragment starts at position 4 and the shortest at position 15. A peptide starting at position 7 appears as the major component of non-(1-84) PTH fragments. The generation process is similar to the one described for smaller C-PTH fragments a number of years ago, suggesting a similar production mechanism and source for all C-PTH fragments.

Key words: parathyroid hormone, primary hyperparathyroidism, non-(1-84) parathyroid hormone, secondary hyperparathyroidism, calcium.

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Non-(1-84) parathyroid hormone (PTH) fragments or amino-terminal (N) truncated PTH fragments are large circulating carboxyl-terminal (C) fragments of PTH with a partially preserved N structure. They differ from other circulating C-PTH fragments (which make up the majority of PTH in circulation) by their capacity to react in intact (i) or (13-34) PTH assays [1–3]. Non-(1-84) C-PTH fragments were initially described during a study of parathyroid function in normal individuals [1]. When circulating PTH molecular forms were fractionated by high-performance liquid chromatography (HPLC) at various calcium concentrations, a slightly less hydrophobic peak of immunoreactivity was identified in front of hPTH (1-84) and named non-(1-84) PTH. This peak represented about 20% of iPTH in a normal individual under normocalcemic condition but up to 50% in patients with terminal renal failure [1–3]. Like other C-PTH fragments [4], non-(1-84) PTH fragments are cleared by the kidney and thus accumulate in renal failure patients proportionally to the degree of renal failure [5]. They are also secreted by the parathyroid glands [6] and generated during the peripheral metabolism of hPTH (1-84) [6]. The exact N-terminal structure of non-(1-84) PTH fragments is unknown but a recent immunologic study based on the use of antibodies with proximal or distal epitopes in the region (13-34) of the PTH structure suggests that all

non-(1-84) PTH fragments start their N structure prior to position 19 [7].

Our interest in non-(1-84) PTH fragments lies in the fact that they could exert biologic effects different from those of hPTH (1-84). hPTH (7-84), a commercially available surrogate for all non-(1-84) PTH fragments, was initially used to demonstrate that such fragments reacted in iPTH assays [2, 3]. It has been also used to demonstrate that this category of C-PTH fragments exerts biologic effects different from those of hPTH (1-84) via a C-PTH receptor [8–11]. hPTH (7-84) causes hypocalcemia, hypophosphatemia, and antagonizes the calcemic response to hPTH (1-34) and hPTH (1-84) in parathyroidectomized rats [8, 9]. It is also an inhibitor of bone resorption in vitro [11] and of bone turnover in parathyroidectomized 5/6 nephrectomized rats [10]. It is thus important to determine the N-terminal structure of non-(1-84) PTH fragments to see if they could duplicate the biologic effects observed with hPTH (7-84).

METHODS

Experimental subjects

Three patients with primary hyperparathyroidism and three others with secondary hyperparathyroidism, who, upon investigation, had a large amount of parathyroid tissue, were recruited for this project. The study was approved by the Research Ethics Committee of our center, and all participants signed an informed consent.

Experimental protocols

Blood was obtained from each subject prior to surgery to measure indices of calcium metabolism and to perform HPLC separation of circulating PTH molecular forms. Parathyroid tissue taken at surgery was brought to pathology where a portion was kept for pathologic analysis and another was used for experimentation. Five hundred milligrams of tissue proved necessary for successful experimentation with parathyroid cells.

Biochemical measurements

Ionized calcium (Ca^{++}) was measured by a specific electrode (Rapid Lab 348) (Bayer, Toronto, Ontario, Canada), while total calcium (Ca_t), phosphorus (PO_4), creatinine, and alkaline phosphatase (AP) were quantified by automated colorimetric methods. PTH was measured in serum and in HPLC fractions by two enzyme-linked immunosorbent assays (ELISAs) provided by Immutopics Inc. (San Clemente, CA, USA), the Human Bioactive PTH ELISA and the Human PTH ELISA. Both assays use a capture antibody purified by affinity chromatography against hPTH (39-84). The Human Bioactive PTH ELISA has a (1-4) epitope [12]. The revealing antibody of the Human PTH ELISA was purified by affinity chromatography against hPTH (13-34). We also developed a carboxyl-terminal radioimmunoassay (RIA) with a (79-84) immunoaffinity purified antibody provided by Immutopics Inc. and ^{125}I [tyr^{53}] hPTH(53-84) as tracer. The antibody was first purified by affinity chromatography against hPTH (39-84) and further purified by the same method against hPTH (79-84). The behavior of each assay was studied using appropriate hPTH standards. These included hPTH (1-84), hPTH (7-84), [tyr^{34}] hPTH (19-84), [tyr^{34}] hPTH (24-84), hPTH (39-84), hPTH (53-84), hPTH (64-84), and hPTH (53-83). Saturation analysis of the revealing antibody of the two PTH ELISAs was also performed with hPTH (1-34), hPTH (13-34), and hPTH (18-48). All peptides were purchased from Bachem (Torrance, CA, USA) except for hPTH (53-83), [tyr^{34}] hPTH (19-84), and [tyr^{34}] hPTH (24-84) which were generously provided by Dr. H. Jüppner of the Massachusetts General Hospital in Boston.

Parathyroid cell incubation

Parathyroid tissue obtained at surgery was separated from fat and connective tissue, and minced into small pieces. These pieces were digested with collagenase type I (Worthington, 239 U/mg) and DNase type I (Worthington, 3.08 U/mg) [13]. The first was used at a concentration of 20 mg/200 mg of tissue while the second was used at 500 µg/200 mg. Both were dissolved in Dulbecco's modified Eagle's medium (DMEM)/F-12, 10% fetal calf serum (FCS), 0.2% bovine serum albumin (BSA) at 10 mL/200 mg of tissue. Digestion proceeded for 60 minutes at 37°C or overnight at 4°C. The medium was then filtered through a nylon filter (250 µmol/L) and centrifuged at 3500 rpm at 4°C over 10 minutes. After the supernatant was discarded, the pellet was resuspended in digestion medium lacking methionine and washed three times. The cells were then incubated in 10 mL of the same medium in a 25 cm^3 flask at 37°C for 4 hours. Ca^{++} concentration was adjusted to the original Ca^{++} concentration of the patient with $CaCl_2$. Again, after centrifugation, the supernatant was discarded, and the cells were incubated for 8 hours in the same medium to which ^{35}S-methionine 50 µCi/mL had been added with a cocktail of protease inhibitors (Complete, EDTA-free) (Roche P8340). At the end, the medium was centrifuged and the supernatant containing the secreted peptides was kept for further processing. Up to three 8-hour incubations could be achieved with the same cells. When this was done, the HPLC profiles were qualitatively comparable.

HPLC analysis

Circulating PTH molecular forms from all sera and from parathyroid cell incubations were extracted with Waters Sep-Pak Plus C-18 cartridges, as described by

Bennett et al [14]. One C-18 cartridge was used for each 3 mL of serum or medium. Samples were eluted from the cartridge with 3 mL of 800 mL/L acetonitrile in 1 g/L trifluoroacetic acid. Acetonitrile was evaporated from the eluate with nitrogen, and the residual volume was freeze-dried; then reconstituted in 2 mL of 1 g/L trifluroacetic acid for HPLC analysis. Each 2 mL sample was loaded on a Waters C_{18} μBondapak analytic column [300 × 3.9 mm (inner diameter)] and eluted with a noncontinuous linear gradient of acetonitrile in 1 g/L trifluoroacetic acid. The gradient ranged from 15% to 23% in 25 minutes, 23% to 30% in 5 minutes, and 30% to 33% in 30 minutes. The gradient was delivered at 1.5 mL/min with a Hitachi Model L-6200 solvent delivery system. The 1.5 mL fractions were evaporated, freeze-dried, and reconstituted to 1 mL with 7 g/L BSA in water; adequate volumes were then measured in the various PTH assays. Controls experiments were performed with hPTH (1-84) added to hypoparathyroid serum and with internally labeled hPTH (1-84) added to a parathyroid cells incubation experiment where ^{35}S-methionine was omitted to insure that PTH degradation did not occur during the various procedures. After HPLC separation, a single peak of radioactivity coeluting with hPTH (1-84) was seen and single peak of immunoreactivity coeluting with hPTH (1-84) was detected by the two PTH assays. Immunoreactive PTH recovery with the two PTH ELISAs through all of these procedures was calculated by comparing original serum or medium PTH value with the sum of PTH immunoreactivity across all HPLC fractions. For the hPTH (79-84) assay, this was accomplished by comparing the amount of immunoreactivity recovered by this assay with that of the two other PTH assays.

Radioactive protein sequencing

Localization of the metabolically labeled residues was determined by automated amino-terminal Edman degradation, using Applied Biosystems sequenator (Model Procise 494 cLC) (Foster City, CA, USA) operated according to the manufacturer's protocol. The only modification was the addition of narrow-bore tubing linking the outlet of valve 39 to a programmable fraction collector. In the sequencing program, all steps involving transfer to the conversion flask as well as functioning of the conversion flask were eliminated or disabled. Transfer of the butyl chloride (S3) extract was done directly from the reaction cartridge to the fraction collector with functions 119 and 122 each activated for a 45-second duration. Finally, prior to initiating sequencing, two complete degradation cycles, where all additions of phenylisothiocyanate (PITC, R1) were omitted to condition the sample, were programmed, followed by a further cycle with no trifluoroacetic acid addition, permitting double coupling of PITC to the amino terminus in the first cycle. The HPLC-purified and vacuum-dried sample was redissolved in 50 μL of water containing 1 μL of an apomyoglobin solution (75 pmol/μL). The reconstituted sample was then serially added (7 μL at a time) on the 6 mm glass fiber filter on which 7.5 μL of standard Biobrene solution had previously been added. Radioactivity at each cycle was measured after mixing each S3 extract with 3.0 mL of scintillation liquid cocktail (UniverSol, ICN Biomedicals, Irvine, CA, USA) for 10 minutes' duration in Beckman Counter Model LS-8100.

Results are means ± SD. Planimetric evaluation of the various HPLC profiles was accomplished with Origin 7.5 software (Origin Lab Corporation, MA, USA). All HPLC profiles are corrected to 100% recovery and to the patient basal PTH value expressed in pmol/L.

RESULTS

Figure 1 illustrates the immunoreactive characteristics of the three PTH assays used in this study. The Human Bioactive PTH ELISA Kit reacted only with hPTH (1-84) and not with hPTH (7-84). Its revealing antibody could be saturated with hPTH (1-34) but not with hPTH (13-34) or hPTH (18-48). The Human PTH ELISA reacted equally well with hPTH (1-84), hPTH (7-84), half as well with [tyr^{34}] hPTH (19-84) and not at all with other C-PTH fragments, including [tyr^{34}] hPTH (24-84). Its revealing antibody could be completely saturated with hPTH (1-34), about 80% with hPTH (13-34) but only 50% with hPTH (18-48). The hPTH (79-84) RIA reacted slightly better with hPTH (39-84) than with hPTH (1-84), hPTH (7-84), or hPTH (53-84), the latter three being similarly reactive. hPTH (53-83) was only about half as reactive as hPTH (53-84) while hPTH (64-84) was even less reactive.

The biochemical and demographic characteristics of the six patients investigated in this study are depicted on Table 1. Patients with primary hyperparathyroidism had more severe hypercalcemia and were hypophosphatemic. Their AP levels varied from the upper normal limit to frankly elevated concentrations while their PTH levels were moderately elevated. Patients with secondary hyperparathyroidism had normal or slightly elevated calcium levels while being hyperphosphatemic. Their AP levels were higher than those of patients with primary hyperparathyroidism and their PTH levels were frankly elevated.

Figure 2 illustrates the HPLC profiles of circulating and parathyroid cell-secreted PTH in a patient with primary (left) and secondary (right) hyperparathyroidism. Three regions of interest were identified by the two PTH ELISAs. The first, region 38 to 42, corresponded to the elution position of hPTH (1-84) and was recognized equally well by both PTH assays. The second, region 32 to 35, corresponded to an amino-terminal form of PTH (N-PTH) which reacted less in the hPTH (12-23) assay

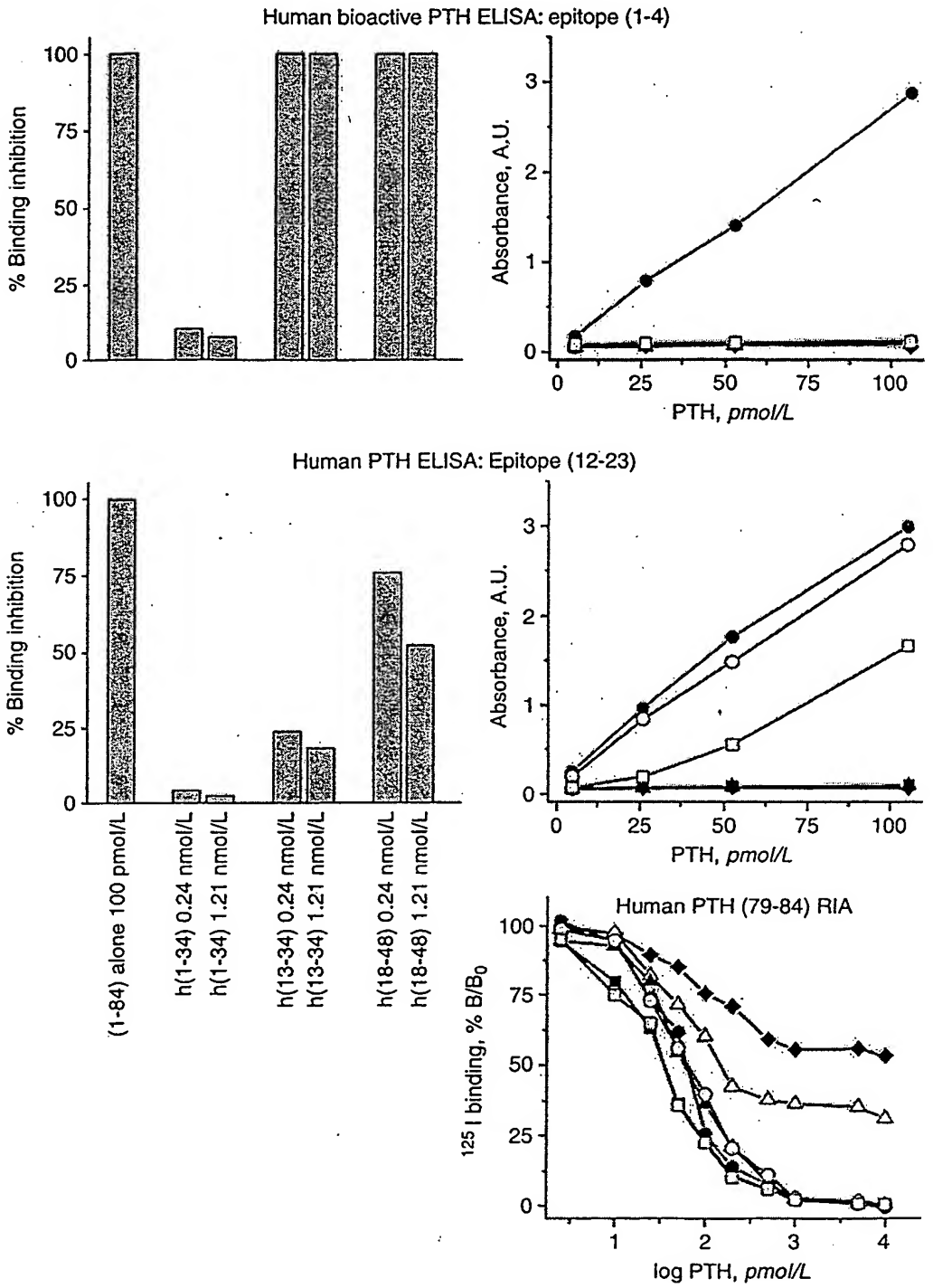

Fig. 1. Immunoreactivity of the three parathyroid hormone (PTH) assays defined with the use of various human PTH calibrators and through saturation analysis of the revealing antibody. (•) hPTH (1-84); (○) hPTH (7-84); (■) hPTH (39-84); (▲) hPTH (53-84); (△) hPTH (53-83); (◆) hPTH (64-84); (□) [tyr^{34}] hPTH (19-84); (*) [tyr^{34}] hPTH (24-84). AU is arbitrary units.

in most HPLC profiles. Finally, the third region, region 21 to 28, corresponded to non-(1-84) PTH fragments and reacted better in the hPTH (12-23) assay. This region was arbitrarily divided into two regions, 3 (21 to 25) and 4 (26 to 28), for sequence analysis because region 3 had significant hPTH (1-4) immunoreactivity while region 4 almost none.

Table 2 summarizes the planimetric evaluation of HPLC profiles performed on serum or parathyroid cells supernatant. The percent of immunoreactivity

Table 1. Characteristics of patients with primary hyperparathyroidism (PHP) and secondary hyperparathyroidism (SHP)

			Biochemical measurements						
Groups	Number	Gender	Ca^{++} (1.19-1.34 mmol/L)	Ca_t (2.11-2.56 mmol/L)	PO_4 (0.77-1.4 mmol/L)	Creatinine (38-115 μmol/L)	Alkaline phosphatase (16-101 U/L)	PTH (1-4) (0.5-4.1 pmol/L)	PTH (12-23) (1.5-7.0 pmol/L)
PHP	1	F	1.88	2.88	0.47	56	172	24.4	42.5
	2	F	1.7	3.19	0.67	73	104	17.6	18.8
	3	M	1.84	3.02	0.57	91	116	24.2	33.5
Mean ± SD			1.81	3.03	0.57	73	130.7	22.1	31.6
			0.09	0.16	0.10	18	36.30	3.9	12
SHP	1	M	1.35	2.32	1.55	654	346	117.8	176.6
	2	M	1.21	2.22	1.73	781	163	102.2	114.5
	3	M	1.3	2.41	2.14	573	129	100.4	166.6
Mean ± SD			1.29	2.32	1.81	669	212.7	106.8	152.6
			0.07	0.10	0.30	105	116.7	9.6	33.3

corresponding to hPTH (1-84), N-PTH, and non-(1-84) PTH regions are given as well as the% immunoreactivity recovery with the two PTH assays in each HPLC profile. The last was better than 80% for both PTH assays and in all profiles, one excepted at 71.9%. With the hPTH (12-23) assay, there was a relatively good concordance between the % of each HPLC region in serum and parathyroid cells supernatant in both populations, with the exception of N-PTH which was higher in the parathyroid cells supernatant of patients with secondary hyperparathyroidism than it was in serum. Furthermore, the percentage of non-(1-84) PTH fragments was similar in serum and parathyroid cells supernatant in both populations. With the PTH (1-4) assay, the amount of N-PTH varied greatly among patients with primary hyperparathyroidism, from 3.3% to 45.5% in serum and from 4.5% to 38.5% in parathyroid cells supernatant, affecting other region results. This problem still existed but to a lesser extend in patients with secondary hyperparathyroidism.

The ^{35}S-methionine counts corresponding to HPLC regions 1, 2, 3, and 4 (Fig. 2) were submitted to sequence analysis. The results for one patient with primary hyperparathyroidism and one with secondary hyperparathyropidism are illustrated in Figure 3. These data are also summarized for all patients in Table 3. Peak 1, corresponding to the elution position of hPTH (1-84), disclosed ^{35}S-methionine residues at cycles 8 and 18, corresponding to a single peptide starting at position 1 in all cases. Peak 2 gave similar results in all cases with a dominant peptide starting at position 1, but also weaker signals at positions 5 and 15 in two cases and 2 and 12 in two other cases, corresponding to peptides starting at positions 4 and 7, respectively. Peak 3 gave signals at positions 8 and 18, 2 and 12, as well as four corresponding to peptides starting at positions 1, 7, and 15. Positions 8 and 18 were the most important quantitatively in four cases while 2 and 12 in two cases. Peak 4 gave signals corresponding to peptides starting at positions 1, 7, 8, 10, and 15. The most important signal corresponded to a peptide starting at position 7 in all cases.

Figure 4 summarizes our effort to study the carboxyl-terminal structure of these peaks, with a RIA that reacted better with hPTH (53-84) than with hPTH (53-83), in a patient with primary hyperparathyroidism as well as in a patient with secondary hyperparathyroidism. The amount of PTH recovered by the hPTH (1-4) and (79-84) assays was similar for peaks 1 and 2. For the non-(1-84) region (regions 3 and 4), the amount of immunoreactivity recovered by the hPTH (79-84) assay was only 61.3% (primary hyperparathyroidism type 2) and 83.6% (secondary hyperparathyroidism type 2) of the amount recovered by the hPTH (13-23) assay.

DISCUSSION

This study was planned to elucidate the N-sequence of non-(1-84) PTH fragments. The importance of these fragments in PTH physiology has been evaluated indirectly through the use of hPTH (7-84), a synthetic surrogate for these fragments (8-11). Recent data suggest that these fragments start prior to position 19 of the PTH structure [7]. We decided to sequence internally labeled non-(1-84) PTH fragments because this has been demonstrated to be the most sensitive method in the past [15–17]. The alternative, that is, sequencing purified nonradioactive non-(1-84) PTH fragments, would have required liters of serum from patients with very high PTH levels and liters of medium from parathyroid cell incubations.

We initially worked with small amounts of parathyroid tissue (less than 500 mg) and soon discovered that the amount of internally labeled secretion products was too small to work with efficiently. This is why we started to identify patients with primary or secondary hyperparathyroidism who had larger tumors to enroll them in the protocol. The more severe biochemical status of these patients reflected the greater amount of parathyroid tissue.

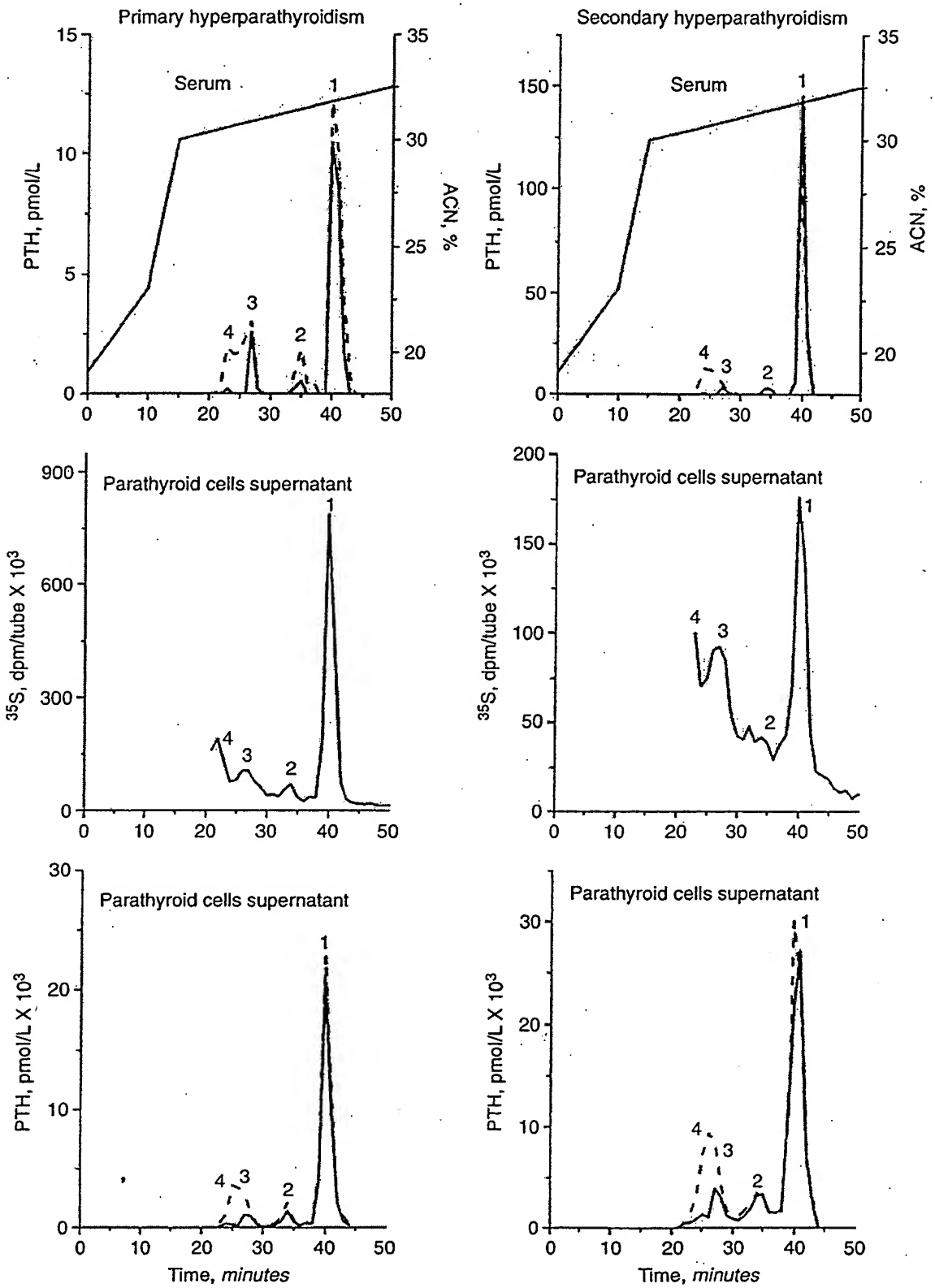

Fig. 2. High-performance liquid chromatography (HPLC) profiles of circulating and secreted parathyroid hormone (PTH) in a patient with primary (left) and a patient with secondary (right) hyperparathyroidism. Immunoreactivity (top and bottom graphs) was analyzed with PTH assays having (1-4) (.....) and (12-23) (-----) epitopes. The middle graph, in each case, represents ^{35}S-methionine counts corresponding to immunoreactivity in the bottom graphs. Regions 1, 2, 3, and 4 corresponding to hPTH (1-84), amino-terminal PTH and non-(1-84) PTH fragments (3 and 4) were submitted to sequence analysis (see Fig. 3).

Table 2. Planimetric evaluation of serum and parathyroid cell medium high-performance liquid chromatography (HPLC) profiles

Milieu	Groups	PTH (1-4) pmol/L	HPLC profile (% of total) (1-84)	N-PTH	Non-(1-84)	Recovery	PTH (12-23) pmol/L	HPLC profile (% of total) (1-84)	N-PTH	Non-(1-84)	Recovery
	PHP 1	24.4	84.5	3.3	12.2	100.9	42.5	62.8	9.1	28.2	82.1
	PHP 2	17.6	62.4	25.0	12.7	85.1	18.8	71.9	4.7	23.4	71.9
	PHP 3	24.2	49.4	45.4	5.2	81.6	33.5	45.1	11.3	43.6	81.1
Serum	Mean ± SD	22.1	65.4	24.6	10.0	89.2	31.6	59.0	8.4	31.7	78.4
		3.8	17.7	21.1	4.2	10.3	12	13.6	3.4	10.5	5.6
	SHP 1	117.8	84.4	9.0	6.6	85.2	176.6	71.0	0.9	28.1	112.9
	SHP 2	102.2	87.7	6.5	5.8	90.3	114.5	69.7	4.7	25.6	95.4
	SHP 3	100.4	84.3	5.9	9.8	94.1	166.6	67.2	0.0	32.8	117.1
	Mean ± SD	106.8	85.5	7.1	7.4	89.9	152.6	69.3	1.9	28.8	108.5
		9.6	1.95	1.6	2.11	4.5	33.3	1.9	2.5	3.7	11.5
Supernatant	PHP 1	2288	91.0	4.5	4.6	99	3078	75.0	5.1	20.0	99
	PHP 2	3413	81.9	13.4	4.7	103	3667	72.9	7.7	19.4	99
	PHP 3	880	59.5	38.5	2.0	101.6	1215	41.2	9.7	49.1	86.9
	Mean ± SD	2194	77.5	18.8	3.8	100.9	2653	63.0	7.5	29.5	94.7
		1269	16.2	17.6	1.5	2	1280	18.9	2.3	17	6.7
	SHP 1	14487	71.2	20.3	8.5	113.1	22109	53.6	19.4	27.0	112.3
	SHP 2	9330	81.4	10.1	8.5	98.5	12600	63.9	8.1	28.0	97.6
	SHP 3	380	86.5	9.9	3.6	100.6	408	76.6	4.5	18.9	97.8
	Mean± SD	8066	79.7	13.4	6.9	104.1	11706	64.7	10.7	24.6	102.6
		7138	7.8	5.9	2.8	7.9	10878	11.5	7.8	5	8.4

Results are means ± SD. Recovery of immunoreactivity in all tubes of the HPLC profile compared to the amount loaded.

The PTH assays used for these studies corresponded to a second generation and a third generation PTH assay. Based on our results and those obtained by another study [12], the Human Bioactive PTH ELISA has a (1-4) epitope its revealing antibody being completely saturable with hPTH (1-34), about 50% with hPTH (2-34), 4% with hPTH (3-34) and less than 0.1% with hPTH (4-34) [12]. The Human PTH ELISA probably has several epitopes in the region (12-23), about 50% of them being proximal to position 18 while 50% distal to position 18. The ending position 23 is based on non reactivity of [tyr^{34}] hPTH (24-84) in the assay while position 12 on the fact that even if hPTH (13-34) was used to immunoaffinity purify the antibody it could not completely saturate the antibody. We also developed a RIA with an imunoaffinity purified (79-84) antibody and ^{125}I [tyr^{53}] hPTH (53-84) as tracer in which hPTH (53-84) reacted better than hPTH (53-84) suggesting an influence of the last amino acid. The fact that hPTH (69-84) was even less reactive than hPTH (53-83) also suggest that the antibody may be sensitive to some tertiary structure element present in hPTH (53-83) but at least partially absent in hPTH (69-84). This assay was mainly used to access the C-terminal structure of non-(1-84) PTH fragments. Although, it does not constitute the perfect tool, it remains the only one available to date to address this question.

The HPLC profiles of circulating and secreted PTH were relatively similar in all patients, with three main regions of immunoreactivity corresponding to hPTH (1-84) (region 1), N-PTH (region 2) and non-(1-84) PTH (regions 3 and 4). The non-(1-84) PTH region was divided into two different regions because hPTH (1-4) immunoreactivity was usually present in region 3 but absent in region 4. Region 1 reacted equally in all three PTH assays and contained a single peptide starting at position 1. The elution position of this peptide was identical to the one of standard hPTH (1-84), indicating similar hydrophobic properties. Region 2 reacted usually better in the hPTH (1-4) and (79-84) assays than in the hPTH (12-23) assay. This is expected because N-PTH is believed posttranslationally modified in region (15-20) [7]. Since the hPTH (12-23) assays has a fair amount of epitopes in that region, one would expect decreased immunoreactivity, which is not complete because epitopes proximal to position 15 and distal to position 20 are also present. The sequence of this peak revealed a major peptide starting at position 1 in all six cases, but also minor peptides starting at position 4 in two cases and at position 7 in two other cases. The last could contribute to some of the immunoreactivity detected by the hPTH (12-23) assay in these patients. Slightly more immunoreactivity was usually detected by hPTH (12-23) and hPTH (79-84) assays than by the hPTH (1-4) assay in region 3. Despite this, the major peptide in that region, the one with the most important ^{35}S-methionine signal, was a peptide starting at position 1 in 4 out of six cases. A peptide starting at position 7 had the most important signal in two cases, while a minor peptide starting at position 15 was also present in all patients. It is difficult to speculate on the structure of the peptide starting at position 1. It is either missing part of the C-terminal end structure or modified posttranslationally differently than N-PTH (peak 2) is. There might be a slight difference between results obtained with the hPTH (79-84) and the hPTH (12-23) PTH assays in favor of the latter, suggesting that all non-(1-84) PTH fragments may not have a structure going to position 84. More data

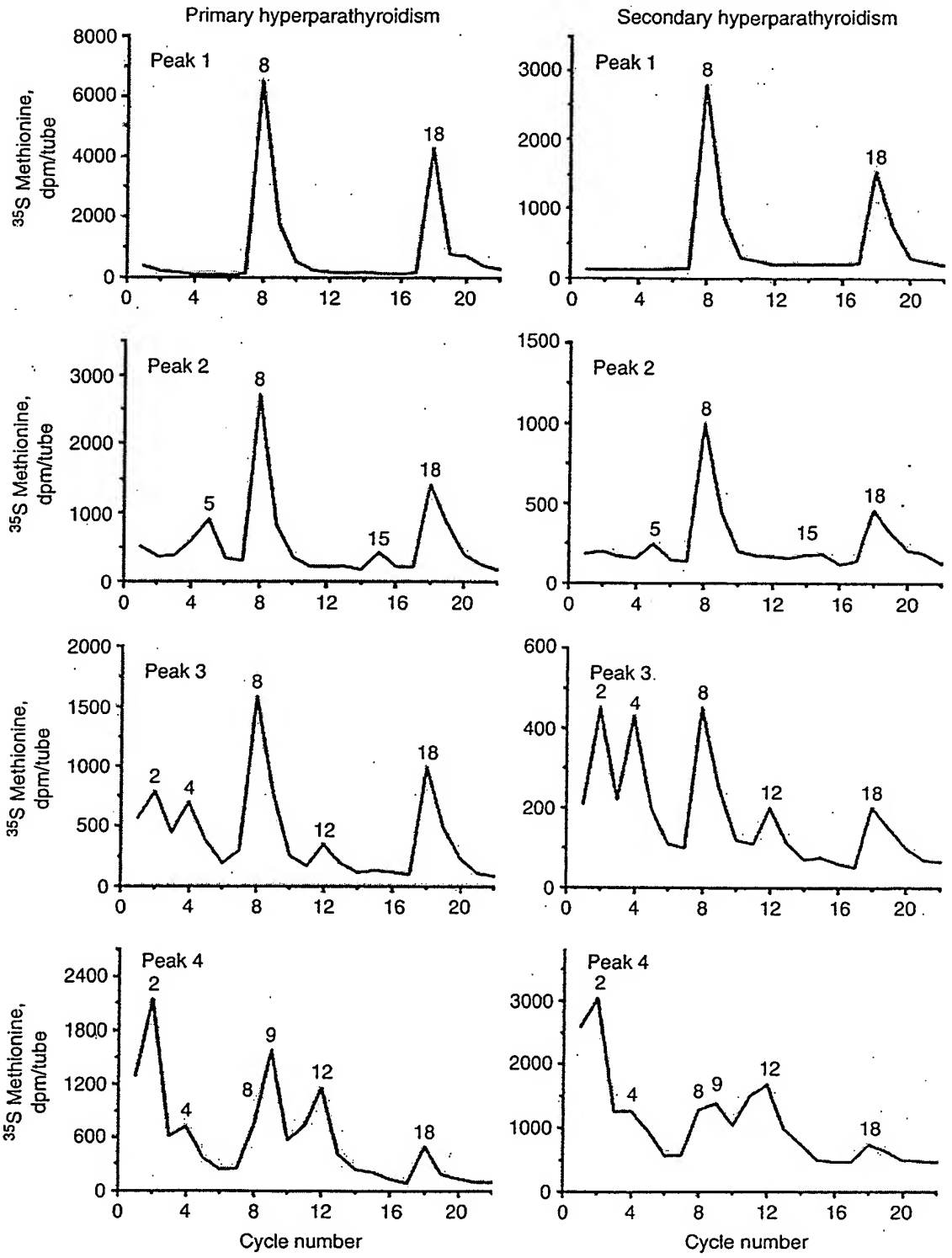

Fig. 3. Sequence analysis of internally labeled parathyroid hormone (PTH) molecular forms secreted by the parathyroid cells of a patient with primary hyperparathyroidism (left) and of a patient with secondary hyperparathyroidism (right). Peaks 1, 2, 3 and 4 refer to immunoreactive peaks identified in Figure 2 and corresponding to hPTH (1-84) (peak 1), N-PTH (peak 2), and non-(1-84) PTH (peaks 3 and 4).

are requested to address this point. Region 4 reacted both with the hPTH (12-23) and (79-84) assays but little with the hPTH (1-4) assay, as expected. Again immunoreactivity with the hPTH (12-23) assay may be slightly more important than with the hPTH (79-84) assay. Sequence analysis revealed the presence of a major signal corresponding to a peptide starting at position 7 in all cases. A minor signal was also present for a peptide starting at

Table 3. Summary of sequence analysis results

HPLC peaks	Sequence analysis of ^{35}S-residues	Starting position	Major signal (no/6)	Signal (no/6)
1	(S)(V)(S)(E)(I)(Q)(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	1	6/6	6/6
2	(S)(V)(S)(E)(I)(Q)(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	1	6/6	6/6
	(E)(I)(Q)(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	4	0/6	2/6
	(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	7	0/6	2/6
3	(S)(V)(S)(E)(I)(Q)(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	1	4/6	6/6
	(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	7	2/6	6/6
	(L)(N)(S)(M)(E)(R)(V)...	15	0/6	6/6
4	(S)(V)(S)(E)(I)(Q)(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	1	0/6	6/6
	(L)(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	7	6/6	6/6
	(M)(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	8	0/6	2/6
	(H)(N)(L)(G)(K)(H)(L)(N)(S)(M)(E)(R)(V)...	10	0/6	3/6
	(L)(N)(S)(M)(E)(R)(V)...	15	0/6	6/6

HPLC is high-performance liquid chromatography. 1, 2, 3, and 4 refer to HPLC peaks identified on HPLC profiles (Figure 2). Major signal indicates the most important signal by quantity of radioactivity. Signal indicates in how many patients studied the signal was present.

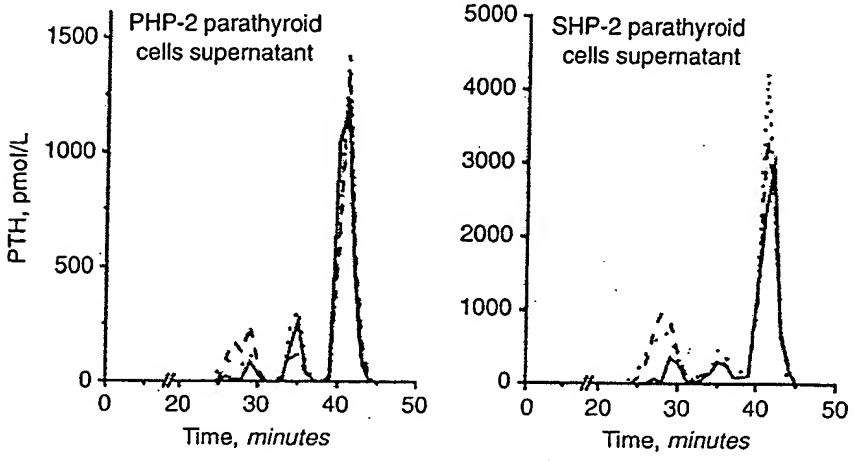

Fig. 4. Immunoreactivity of parathyroid hormone (PTH) molecular forms secreted by parathyroid cells and separated by high-performance liquid chromatography (HPLC) in three different PTH assays with (1-4) (.....), (12-23) (-----), and (79-84) (••••) epitopes.

position 15 in all cases, and for minor peptides starting at position 8 and 10 in two and three cases, respectively. We are very confident of sequence analysis results for peptides having two ^{35}S-methionine signals (those starting at positions 1, 4, 7, and 8) but less for those having a single signal (starting positions 10 and 15). Although unlikely, we cannot completely eliminate the presence of another ^{35}S-methionine labeled peptide interference in the last cases.

Overall, our results suggest that non-(1-84) PTH fragments constitute a family of fragments which have an N-structure starting at positions 4, 7, 8, 10, and 15, the

peptide starting at position 7 being the major fragment. We cannot be specific as to how exactly they are produced. It could be both by specific cleavage at given positions or progressive cleavage from the earlier to the latest position as seen during the peripheral metabolism of various ^{125}I-PTH preparations [4, 15].

The structure of non-(1-84) PTH fragments, as defined here, points to a likely interaction with the C-PTH receptor. This is based on the interaction of hPTH (7-84) and [tyr^{34}] hPTH (19-84) with the C-PTH receptor in various cell lines [8, 9, 18, 19]. It is more than likely that the biological data already obtained with these peptides (8-11) are applicable to the PTH peptides described here. This, in turn, justifies further studies to elucidate the role of these fragments in PTH biology.

ACKNOWLEDGMENTS

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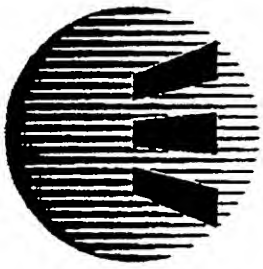


THE ENDOCRINE SOCIETY

Program & Abstracts

Ex. 2

79th Annual Meeting

June 11-14, 1997 Minneapolis, Minnesota

EXHIBIT 2

IMU-3280

P3-194

ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH. JW Colford-[1], M Salvati[1], G MacFarlane[1], LJ Sokoll[2], and MA Levine[2]. [1]INCSTAR Corp., Stillwater, MN 55082; [2]The Johns Hopkins School of Medicine, Baltimore, MD 21205.

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

JWC

#P3-194: ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH.

JW Colford*[1], M Salvati[1], G MacFarlane[1], LJ Sokoll[2], and MA Levine[2]. [1]INCSTAR Corp., Stillwater, MN 55082; [2]The Johns Hopkins Medical Institutions, Baltimore, MD 21205.

IMU-3282

JWL

Introduction

Intact PTH secretion is equisitely controlled by serum ionized calcium in a classic negative feedback loop. Sensitive calcium receptors on parathyroid chief cells detect very slight changes in the concentration of Ca^{2+} and secrete intact PTH in response to low concentrations. Total serum calcium exists in three phases in dynamic equilibrium: protein bound (mainly albumin, Ig), complexed (phosphate, bicarbonate, citrate), and ionized calcium.

The clinical utility of a PTH measurement, in combination with a serum calcium determination, is in diagnosis of primary hyperparathyroidism, management of uremic or seconday hyperparathyroidism, and diagnosis of hypoparathyroidism.

Previously characterized serum PTH heterogeneity includes the intact PTH (1-84) active peptide hormone, and inactive C-terminal fragments with N-termini between PTH (34-43). Thus, strategies for immunoassay evaluation of intact PTH concentrations in serum involved a solid phase antibody present in excess to overcome interfering C-terminal fragments and thus detecting intact PTH only using a N-terminally directed reporter antibody.

N-Terminal truncations yielding PTH species larger than PTH 34-84 have been isolated from human parathyroid cell monolayer.C-terminal fragments have been detected in extracts of parathyroid gland, and parathyroid effluent samples collected from hypercalcemic patients with hyperparathyroidism. Based on immunoassay value differences observed by our group and PTH immunoreactivity by Brossard et.al. (JCEM 81(11): 3923-3929) there appears to be differences in immunoassay values caused by fragment recognition in some commercially available intact PTH assays.

Evidence for a circulating inhibitor to PTH has arisen from studies of patients with pseudohyperparathyroidism. Despite elevated levels of immunoreactive PTH, bioactive PTH was normal when tested in bioassay systems. Plasma from these patients has been shown to diminish the biological activity of exogenous PTH in these *in vitro* bioassays.

To determine what PTH molecular forms circulate, and elucidate possible biological activity, a detection system was developed to elucidate the concentrations of intact PTH and the novel large molecular weight fragments of PTH, without interference or recognition of the hepatically generated C-terminal fragments. Data generated was evaluated for diagnostic potential with regard to primary hyperparathyroidism, and correlation to serum calcium in primary and uremic hyperparathyroidism.

IMU-3283

JJC

Abstract

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

JWC

PTH Values inUremic Hyperparathyroidism

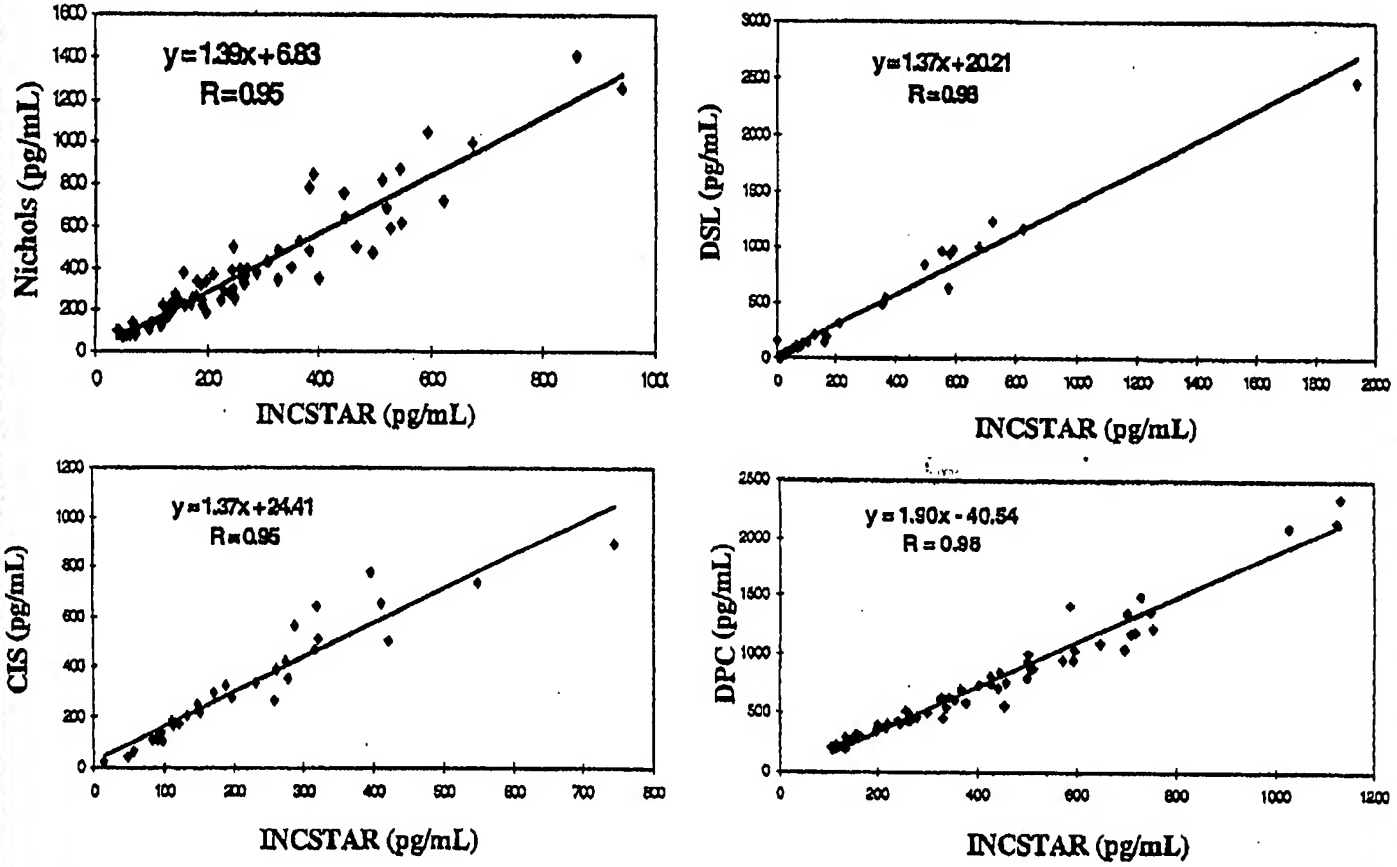


Figure 1. Correlations of several commercially available intact PTH assays verses the INCSTAR N-tact PTH SP intact PTH assay. All of the commercial assays evaluated indicate much greater cross-reactivity to the novel PTH fragments.

IMU-3285

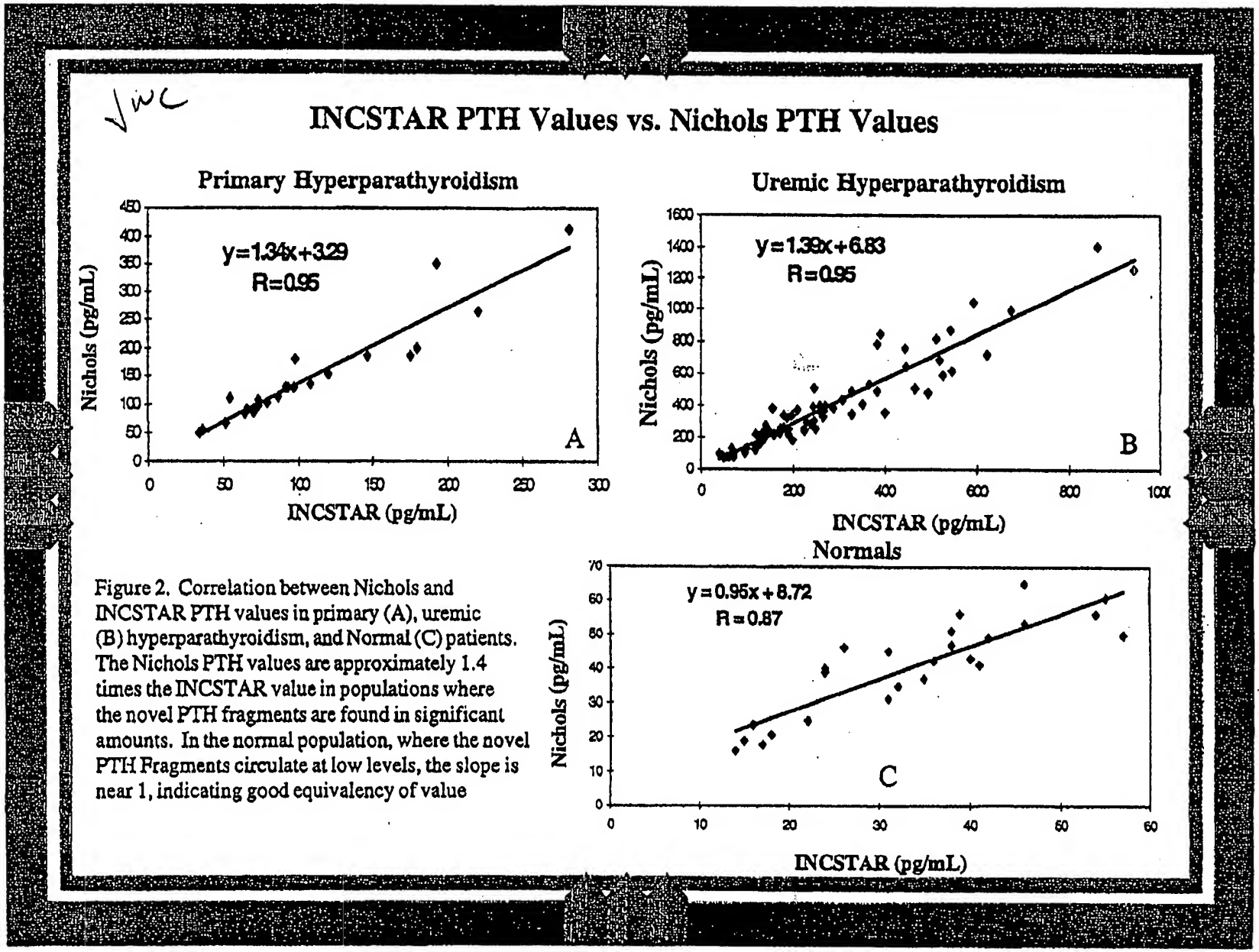
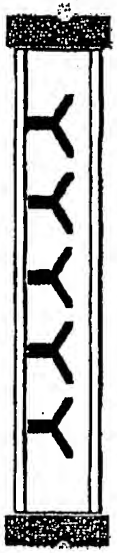


Figure 2. Correlation between Nichols and INCSTAR PTH values in primary (A), uremic (B) hyperparathyroidism, and Normal (C) patients. The Nichols PTH values are approximately 1.4 times the INCSTAR value in populations where the novel PTH fragments are found in significant amounts. In the normal population, where the novel PTH Fragments circulate at low levels, the slope is near 1, indicating good equivalency of value

Method of Isolation of PTH Molecular Forms



Delipidated EDTA plasma is loaded on the column. PTH Molecular forms containing all or parts of the (39-84) region are captured. The specifically bound protein is eluted with 0.2 M glycine pH 2.5

Anti-PTH(39-84) Immunoextraction Column

The protein eluted from the immunoextraction step is loaded onto a C_{18} reverse-phase HPLC column. The column resolves homologous proteins by size. 2-60% - 0.1% TFA/Acetonitrile : 0.1% TFA/dH20 over 58 minutes 1%/minute.

C_{18} Reverse Phase HPLC Column

HPLC Fractions were tested for PTH immunoreactivity

Figure 3. Method of isolation of PTH molecular forms.

IMU-3287

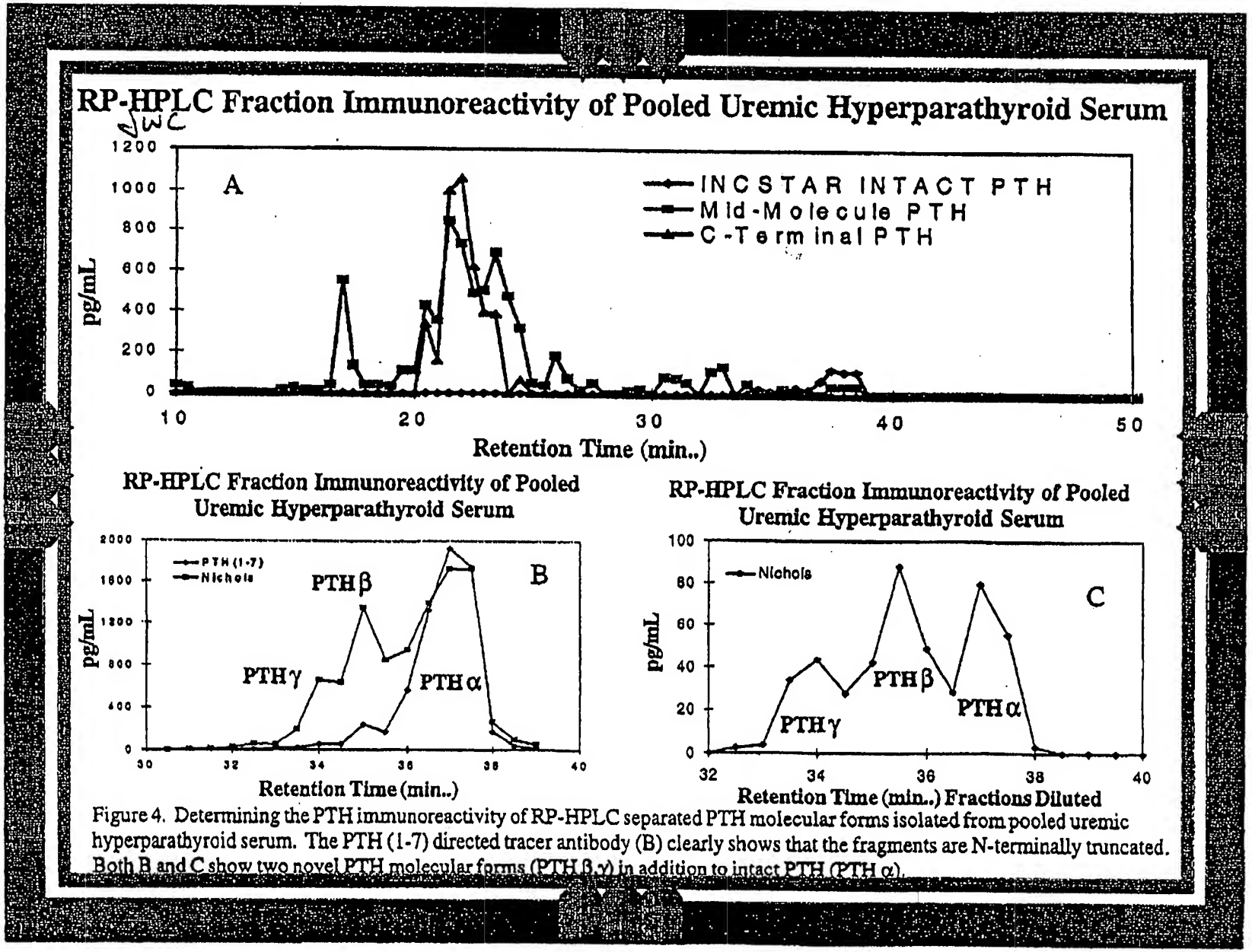


Figure 4. Determining the PTH immunoreactivity of RP-HPLC separated PTH molecular forms isolated from pooled uremic hyperparathyroid serum. The PTH (1-7) directed tracer antibody (B) clearly shows that the fragments are N-terminally truncated. Both B and C show two novel PTH molecular forms (PTH β,γ) in addition to intact PTH (PTH α).

IMU-3288

JWC

PTH Molecular Forms

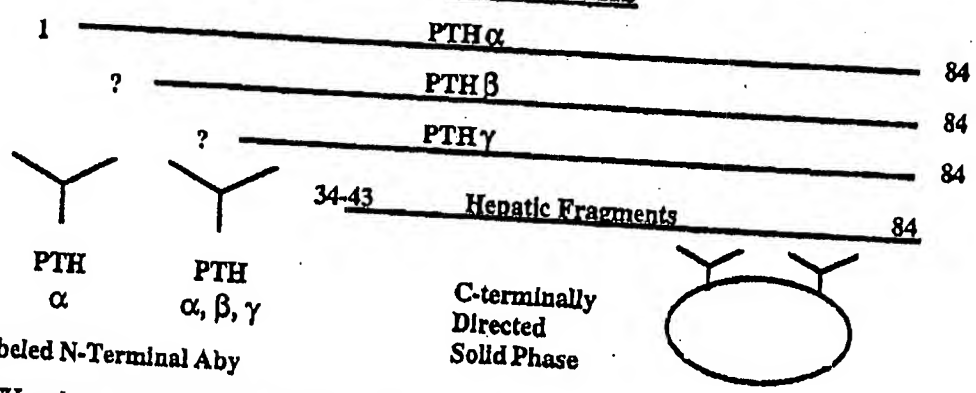


Figure 5. Defining PTH molecular forms isolated by RP-HPLC and the detection system used to estimate their concentration. These fragments have N-termini that extend beyond amino acid 34, and do not include hepatically generated fragments.

PTH Ratio

$$\frac{[\text{PTH } \alpha, \beta, \gamma] - [\text{PTH } \alpha]}{[\text{PTH } \alpha]} = \frac{\text{PTH } \beta,\gamma}{\text{PTH } \alpha}$$

This calculation yields a ratio of the two novel C-terminal Fragments / Intact PTH

The hepatically generated C-terminal fragments are NOT evaluated in this system

Figure 6. Defining the PTH Ratio to be the concentrations of the two novel PTH fragments to the concentration of intact PTH

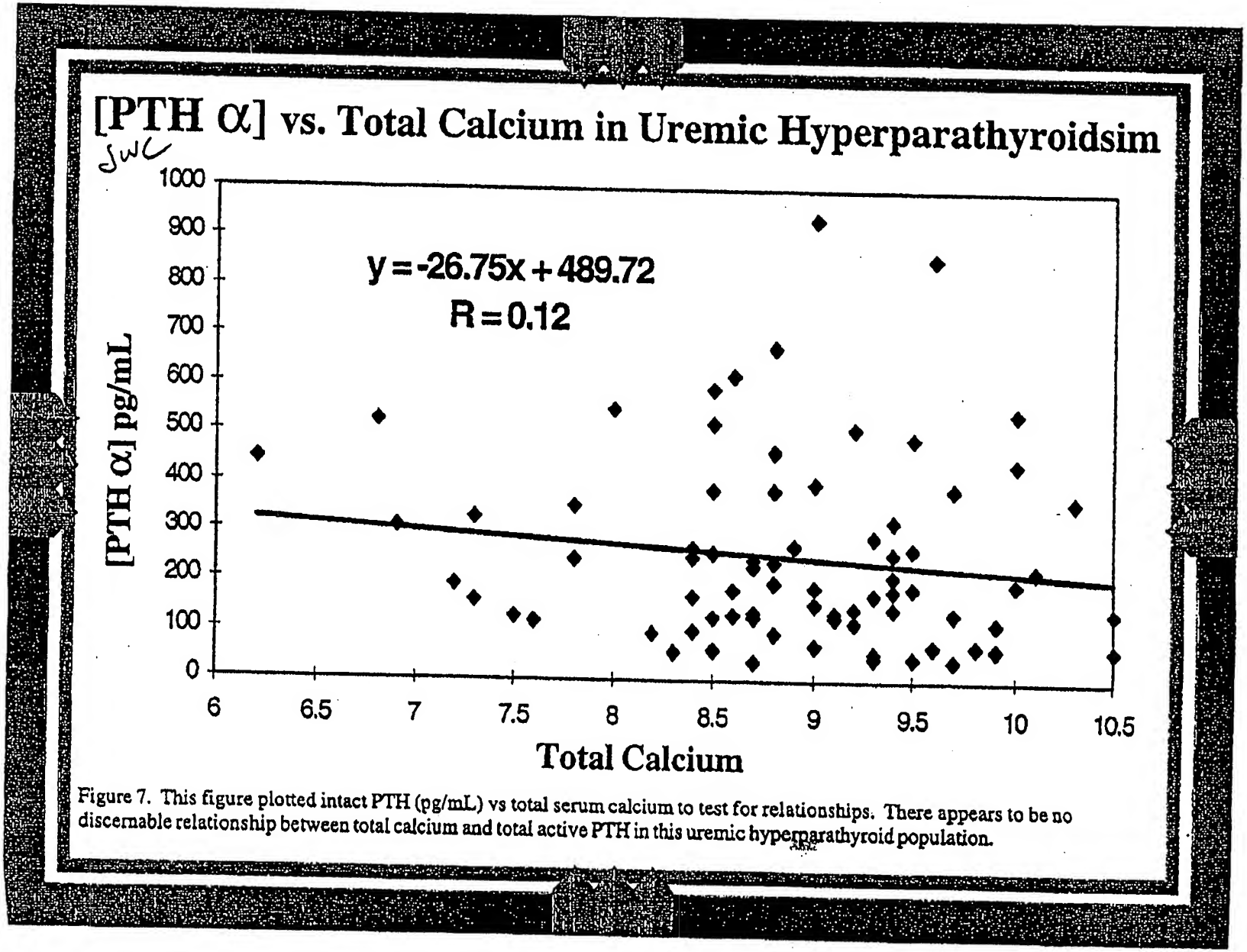


Figure 7. This figure plotted intact PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernable relationship between total calcium and total active PTH in this uremic hyperparathyroid population.

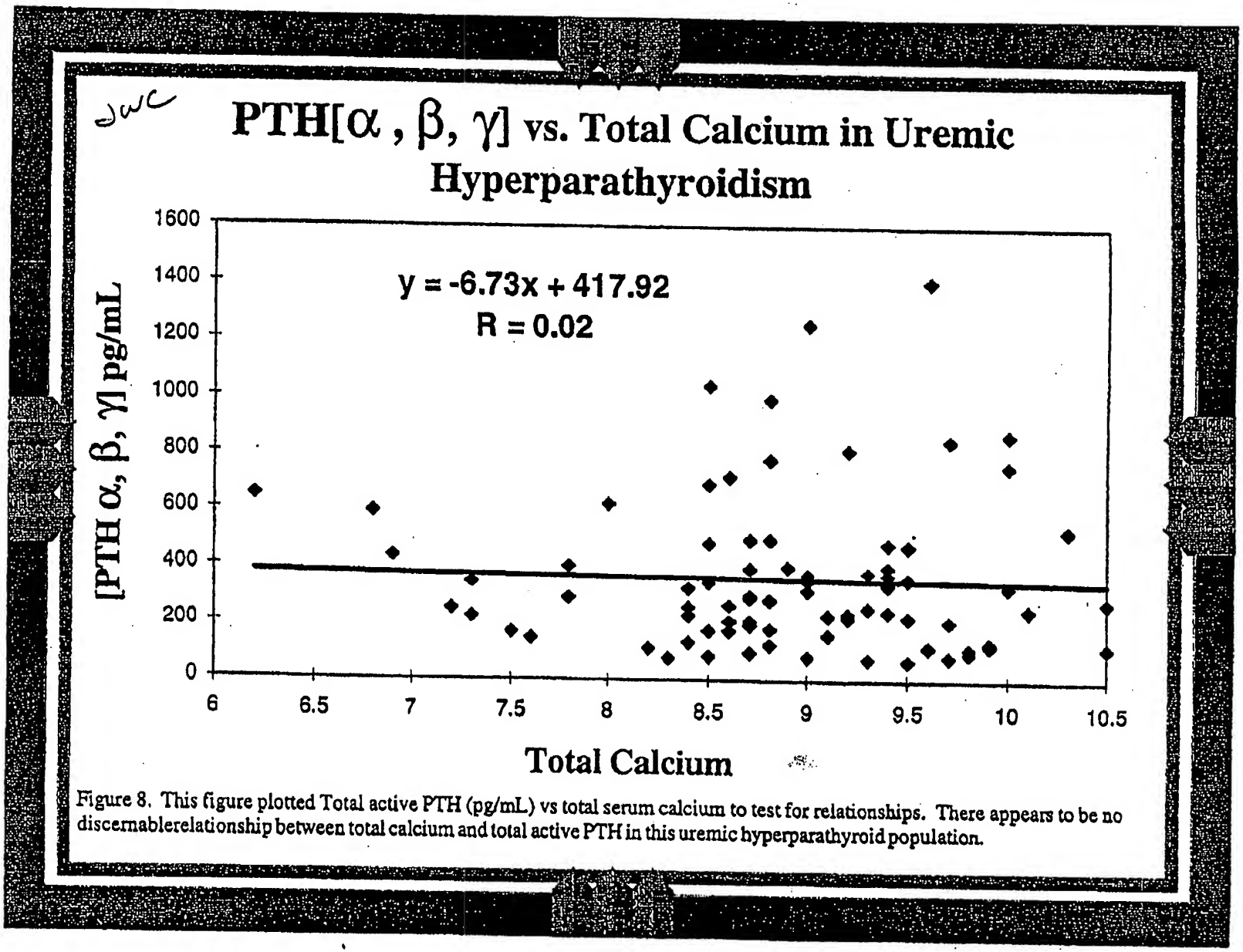


Figure 8. This figure plotted Total active PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernablerelationship between total calcium and total active PTH in this uremic hyperparathyroid population.

IMU-3291

PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Uremic Hyperparathyroidism

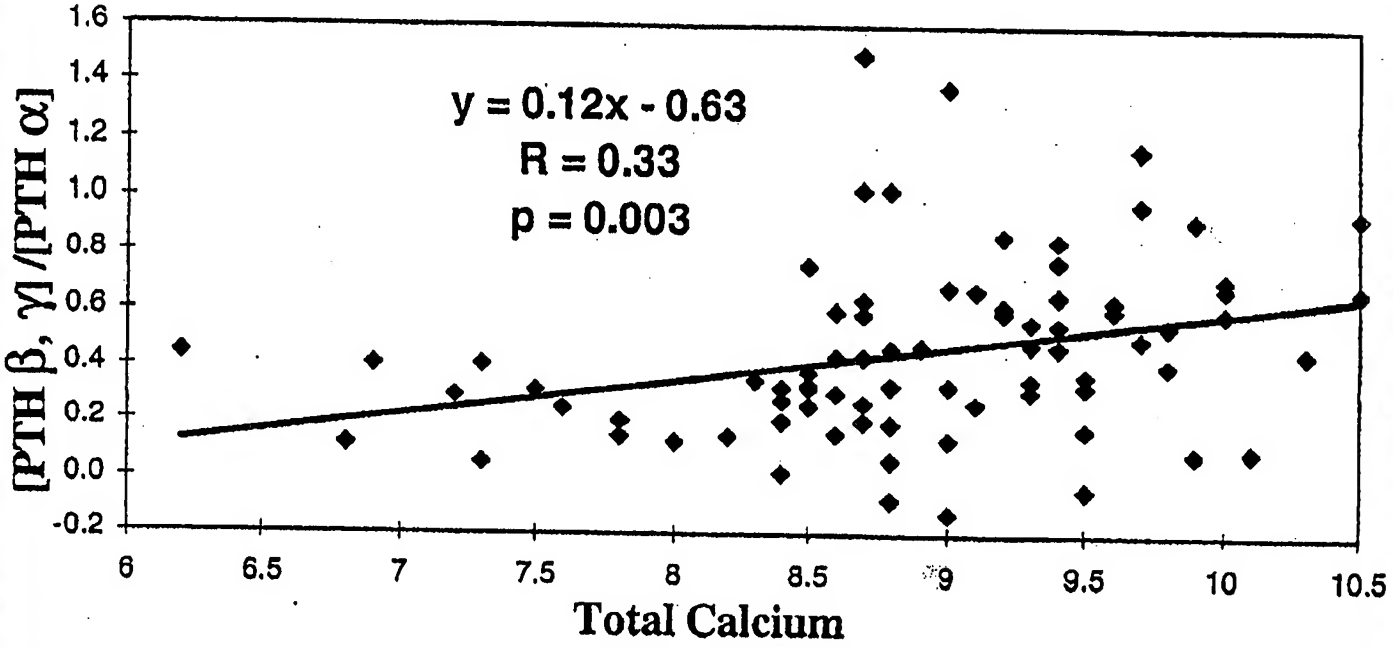


Figure 9. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, the corresponding serum calcium approaches normalization in uremic hyperparathyroidism even though most of these samples have elevated intact PTH levels. This trend is opposite to that in primary hyperparathyroidism. Endogenous and exogenous factors, such as calcitriol therapy, complicate calcium homeostasis in uremic hyperparathyroidism, and thus cause and effect conclusions cannot be made in this population without more information. However, this is consistent with an inhibitory effect of these fragments to PTH in these patients.

PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Primary Hyperparathyroidism

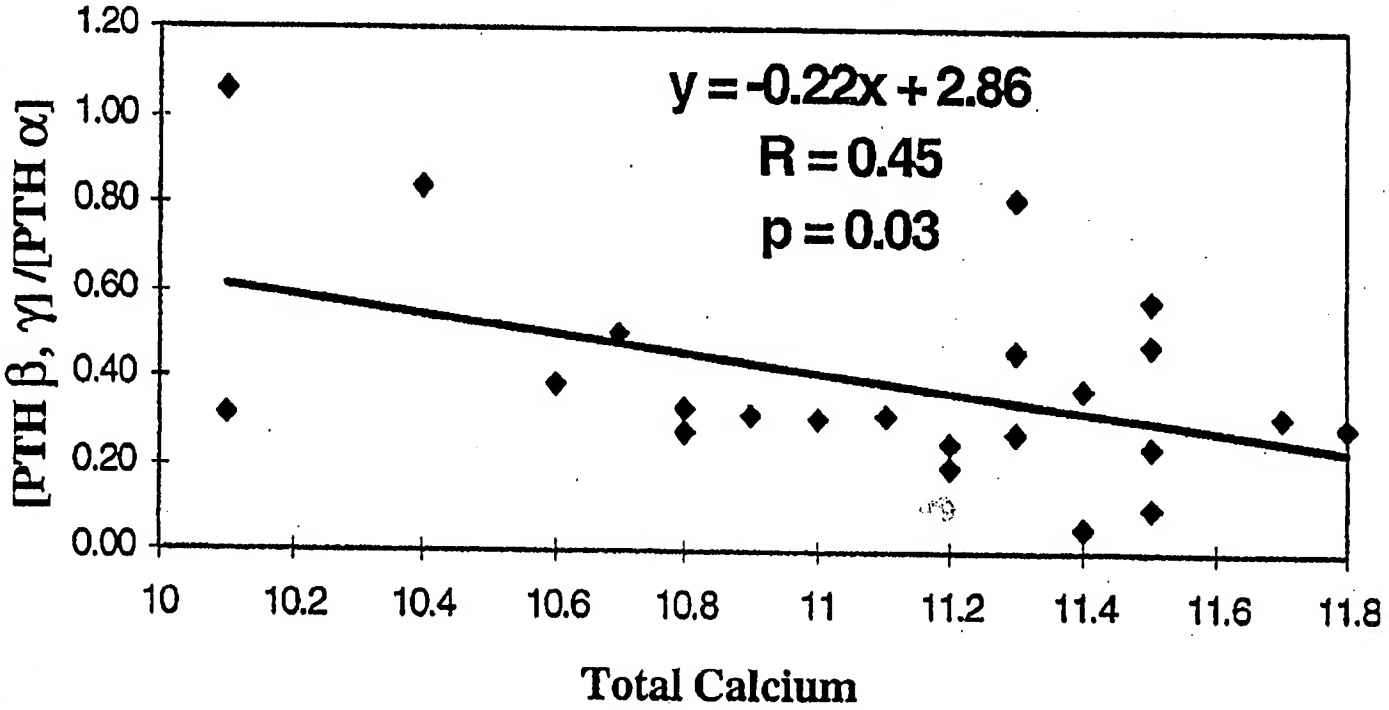


Figure 10. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, there is a trend of normalization of serum calcium in primary hyperparathyroidism even though all of these samples have elevated intact PTH levels. This is consistant with an inhibitory effect of these fragments to PTH action in this population.

IMU-3293

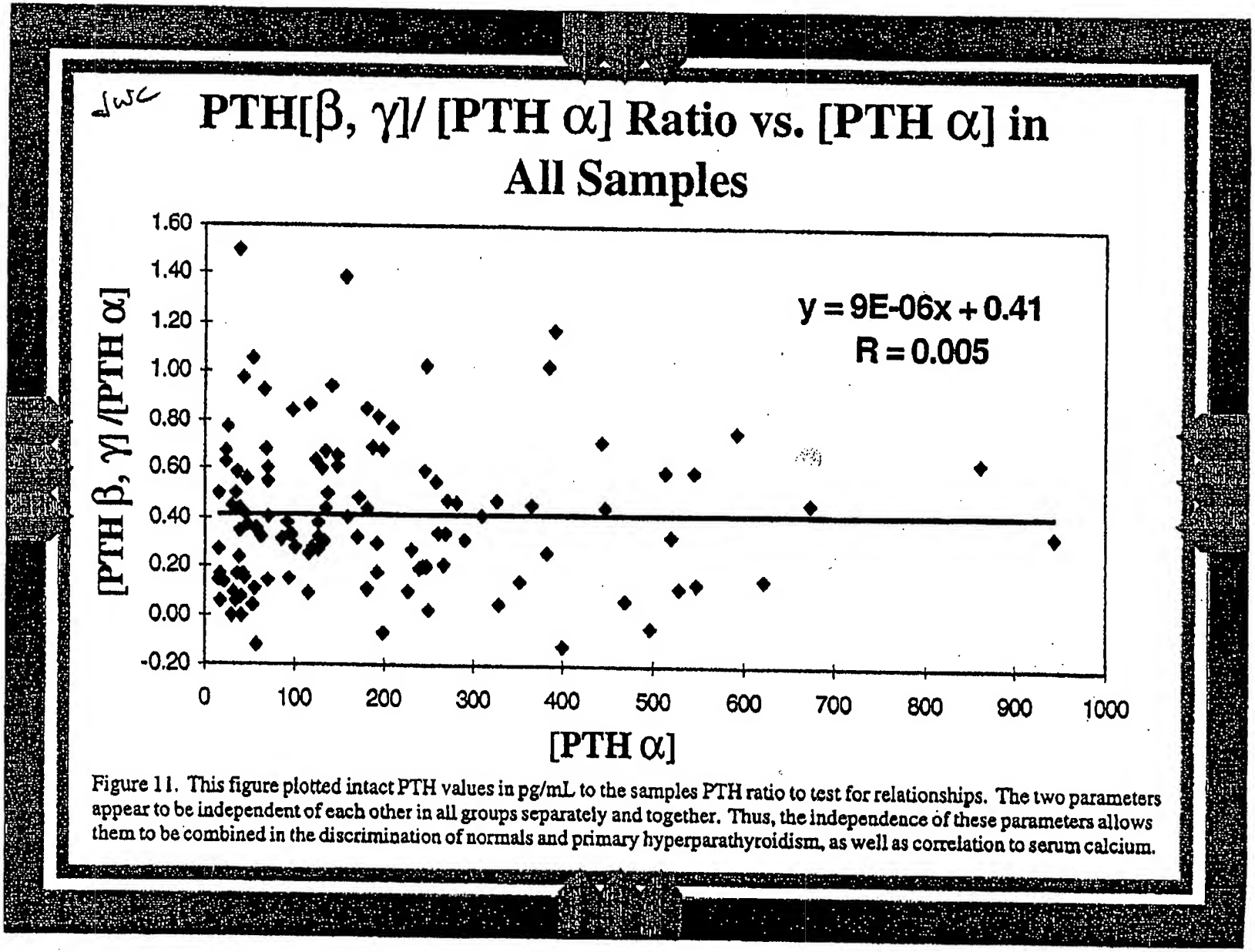
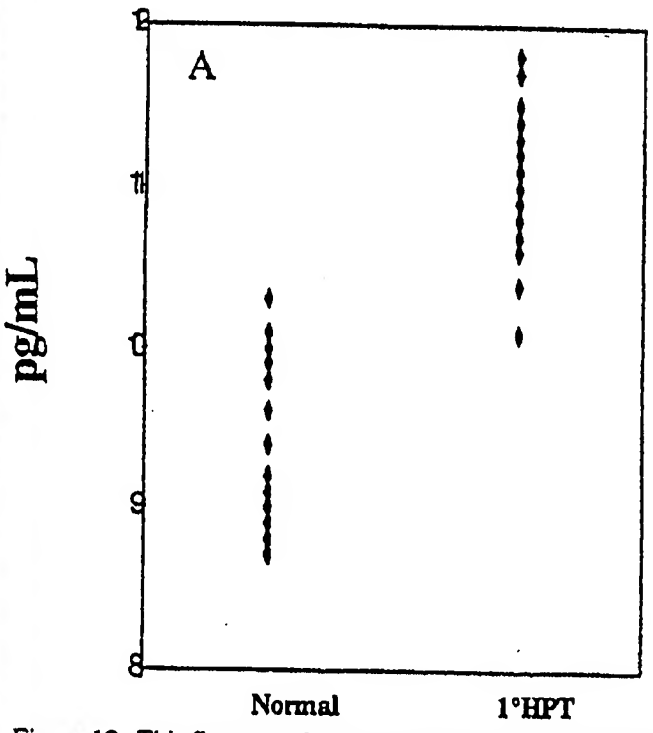


Figure 11. This figure plotted intact PTH values in pg/mL to the samples PTH ratio to test for relationships. The two parameters appear to be independent of each other in all groups separately and together. Thus, the independence of these parameters allows them to be combined in the discrimination of normals and primary hyperparathyroidism, as well as correlation to serum calcium.

JWC

Discrimination Power of Total Calcium



Discrimination Power of PTH

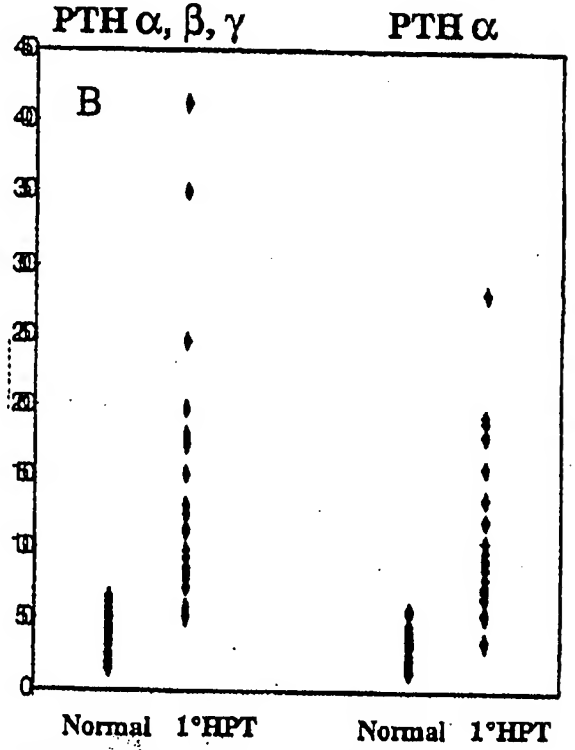


Figure 12. This figure used serum total calcium (A) and PTH values in pg/mL(B) to compare the discrimination power of calcium and PTH measurement in the separation of a normal and primary hyperparathyroid population. There is clearly significant overlap of the populations using both calcium and PTH determinations separately. These parameters are typically combined in a clinical situation to provide optimal discrimination, but which PTH parameter is the best is evaluated in Figure 13.

Discrimination Power of PTH measurements + Total Calcium

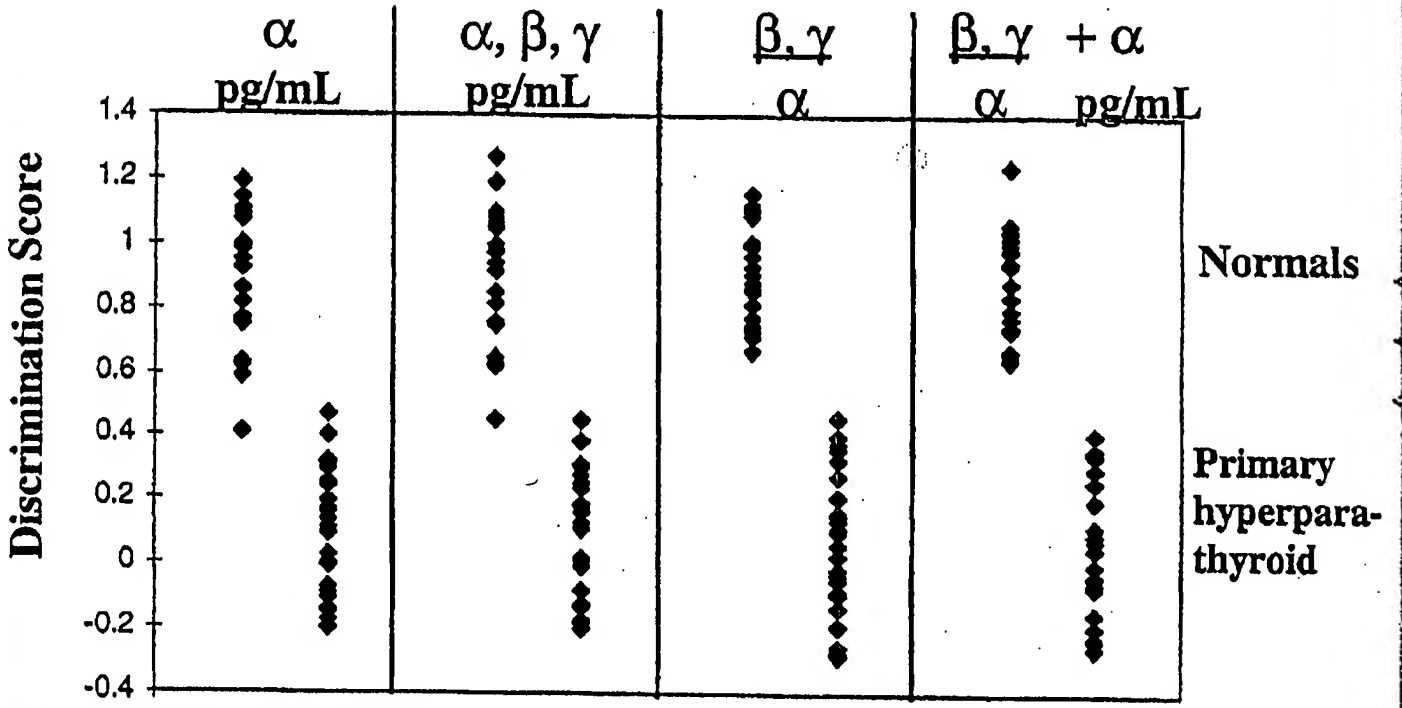


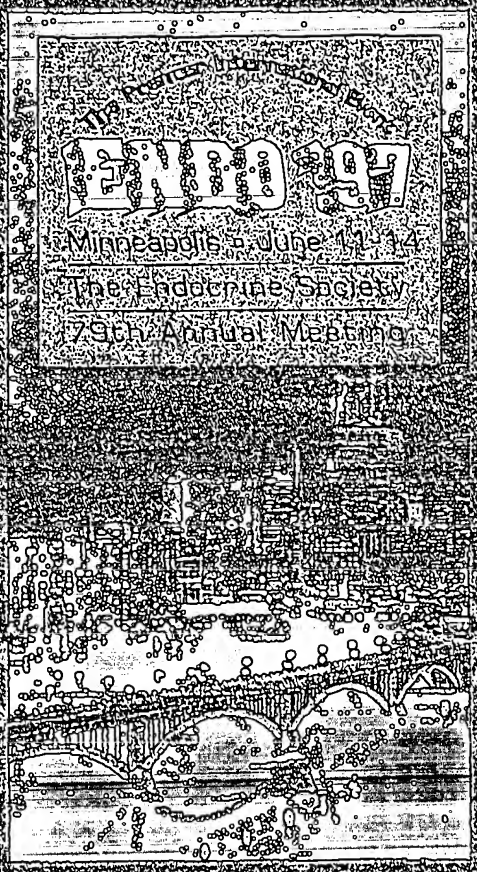
Figure 13. This figure used regression analysis to compare the discrimination power of a PTH measurement in addition to a total calcium determination in the separation of a normal and primary hyperparathyroid population. The combination of a PTH measurement with a serum calcium determination is the common clinical situation in diagnosis of primary hyperparathyroidism. The PTH ratio allows clear distinction of borderline cases into the respective populations.

JWC

CONCLUSIONS

- There appears to be PTH heterogeneity found in circulation in patients with primary and uremic hyperparathyroidism beyond what has been characterized thus far.
- These novel PTH fragments appear to be N-terminally truncated and are reported in some commercially available intact PTH assays.
- As levels of the fragments approach those of Intact PTH, serum calcium trends toward normalization in patients with primary and uremic hyperparathyroidism
- Based on the data and references, we speculate that these fragments are a strong candidate for being a competitive inhibitor to intact parathyroid hormone action at the PTH/PTHrP receptor in patients with primary hyperparathyroidismand mediate its biological activity.
- Complete characterization of PTH molecular forms provides a better correlation to serum calcium, and provides greater clinical discrimination between primary hyperparathyroid and normals.

PROGRAM & ABSTRACTS



79TH ANNUAL MEETING

JUNE 14-17, 1997

MINNEAPOLIS, MINNESOTA

THE ENDOCRINE SOCIETY

P03-194
Friday June 13, 1997
PTH and Calcium [BASIC - Poster Session] (Exhibit Hall 1&2)

Isolation and characterization of large molecular weight fragments of PTH

J.W. Colford[1], M. Salvati[1], G. MacFarlane[1], L.J. Sokoll[2], M.A. Levine[2]

[1] *INCSTAR Corp., Stillwater, MN, USA*
[2] *The Johns Hopkins School of Medicine, Baltimore, MD, USA*

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5 M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

Biochemistry: other
Parathyroid hormone

COMPARING SPECIFICITY FOR INTACT HUMAN PARATHYROID HORMONE BETWEEN INCSTAR PTHSP AND NICHOLS INTACT PTH ASSAYS.

Todd Jensen, Jon Spring, and John Colford (INCSTAR Corporation, Stillwater, MN 55082)

ABSTRACT

The N-terminus of the PTH molecule is required for PTH receptor interaction. It has been shown that cleavage of three amino acids from the N-terminus will inactivate PTH activity *in vivo*. Specificity is an advantage of a two antibody assay format of an IRMA. An N-terminal truncated circulating inhibitor to PTH has been hypothesized, but not found. Both the INCSTAR Intact PTHSP™ assay and the Nichols Allegro™ Intact PTH claim to be specific for the intact molecule. In a comparison between the two kits, patients with renal failure were assayed. The results showed that the Nichols assay results to be an average of 1.52 times the INCSTAR assay value (n = 14). Intact 1-84 PTH was spiked into clarified human serum and assayed in both kits. The results showed that the Nichols assay results to be an average of 1.07 times the INCSTAR assay value (n = 12).

Both assay tracers are directed to the N-terminal region of the intact PTH molecule. To compare specificity of the two assay tracers, we used a (7-84) C-terminal fragment. While not a defined byproduct of PTH metabolism *in vivo*, it could provide insight to the degree the population of antibodies in the tracer are directed to the N-terminus. Based on weight, target levels of 1500, 1000, and 500 pg/ml (7-84) PTH C-terminal fragment were spiked into clarified human serum. the Nichols assay had values of 520, 374, and 245 pg/ml, respectively. The INCSTAR assay had values of 220, 167, and 114 pg/ml, respectively. This showed that the Nichols results to be an average of 2.25 times the INCSTAR assay value (n = 14).

The INCSTAR tracer was substituted into the Nichols assay and the (7-84) PTH spiked serum samples were assayed, with the hybrid assay reporting 258, 191, and 121 pg/ml, respectively. This suggests that the INCSTAR tracer gives the INCSTAR assay greater specificity for the intact PTH molecule than the Nichols assay. These results also suggest the presence of a C-terminal fragment of PTH in renal samples that has not been currently identified.

INTRODUCTION

Recently, it has been reported that there is a non-(1-84) molecular form of PTH found in normals and renal samples that is immunoreactive in the Nichols Allegro™ Intact PTH assay (Brossard et al, Proc. Int. Cong. Endo, Vol2, OR60-5). The PTH molecule is degraded in the parathyroid gland itself and also in the liver. The breakdown products don't have most of the N-terminal 27 amino acids and lack hypercalcemic, hypocalciuric, and phosphaturic activity. These fragments have a considerable half-life and accumulate in circulation up to 20 times the concentration of the intact hormone. Their presence makes the immunologic assessment of the biologically active hormone difficult.

The hepatic fragments are generated when enzymes on the surface of Kupffer cells cleave the hormone in its 34-43 region. These fragments are formed at a rate dependent largely on the concentration of intact PTH. The N-terminal fragment is at very low concentrations in serum, suggesting a quick and thorough removal from circulation. Fragments generated in the parathyroid gland are truncated at both ends of the molecule. These fragments are not currently defined. In hypercalcemic patients of a non-parathyroid origin, the negative feedback mechanism will suppress PTH release to almost nothing, but the parathyroid gland will continue to release truncated fragments. In patients that have nonparathyroid forms of hypercalcemia, these fragments have been detected by a carboxy- terminal RIA but the N-terminus was not defined. All PTH fragments are removed from circulation by the kidney. In cases of renal insufficiency, fragments accumulate.

There appear to be differences in the values for renal samples among the Nichols Allegro™, DSL Active™, and INCSTAR N-tact® PTHSP assays. To explore these differences, PTH (1-84) and (7-84) immunoreactivity was validated between the assays. If PTH (1-84) equivalence is shown, renal sample differences will be significant. The results will be compared to reported non-(1-84) PTH immunoreactivity.

Dilution Linearity Validation

				% Recovery	
Pat. ID#	46	46 1:2	46 1:4	46 1:2	46 1:4
Nichols	1142	573	277	100	97
DSL	1233	707	378	115	123
INCSTAR	725	354	159	98	88
EXP(1-7)	860	383	158	89	74
EXP(7-84)	680	343	158	101	93

			% Recovery
Pat. ID#	48	48 1:2	48 1:2
Nichols	877	485	111
DSL	983	546	111
INCSTAR	593	278	94
EXP(1-7)	613	300	98
EXP(7-84)	533	253	95

			% Recovery
Pat. ID#	49	49 1:2	49 1:2
Nichols	143	67	94
DSL	143	69	96
INCSTAR	107	51	95
EXP(1-7)	102	44	87
EXP(7-84)	104	59	112

PTH(7-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(7-84)
1	272	217	193	238
2	515	395	296	393
3	1262	1168	816	1094
4	1720	1623	1059	1693

$p<$ 0.0004

INCSTAR Tracer is unique
Nichols, DSL, and EXP (7-84)
are not shown different

PTH(1-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)
1	175	168	175	201	182
2	188	184	222	263	218
3	696	772	620	648	615
4	1058	1152	1045	1074	988

$p=$ 0.45

No Observed Difference

PTH Tracer Specificity.

	NSB	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	20	5419	40	1452	3643	3294	8	0	12	14	19
DSL	559	11627	415	5585	8922	6916	556	364	424	319	278
Nichols	195	2982	139	968	2606	2136	153	138	139	220	142

Yes is a signal 2 SD above Background

	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No
DSL	Yes	No	Yes	Yes	Yes	No	No	No	No	No
Nichols	Yes	No	Yes	Yes	Yes	No	No	No	No	No

Values from two separate assays confirming differences in (7-84) Spiked serum values between Nichols and INCSTAR

FRAGMENT(7-84) SPIKES (pg/mL)

	Target Value*	INCSTAR Assay	Nichols Assay	DIFFERENCE
Assay 1	1500	511	774	-262
	1000	366	577	-211
	500	220	346	-125
Assay 2	1000	220	520	-300
	400	167	374	-207
	250	114	245	-131

* - Value based on assumption of 100% purity and quantitative transfer in dilution

MEAN=	-206.0	
Variance=	4835.4	
Standard Error=	28.39	
***t*=**	7.26	
***p*=**	0.00078	Significant difference

Comparison of (1-84) Spiked serum values and controls between Nichols and INCSTAR

		INTACT (1-84) (pg/mL)		
		INCSTAR	Nichols	DIFFERENCE
Serum Spikes	spike 1	2805	2883	-77
	spike 2	1617	1712	-95
	spike 1 Diluted 1:10	273	236	37
	spike 2 Diluted 1:10	157	152	5
	spike 3	1656	1589	67
	spike 4	1740	1724	16
	spike 3 Diluted 1:10	183	212	-29
	spike 4 Diluted 1:10	164	190	-26
Kit Controls	NJ	36	31	6
	NK	265	214	51
	(INC)L1	33	34	-1
	(INC)L2	415	330	85
	(INC)L1	49	41	8
	(INC)L2	447	426	21

MEAN of Ln*=	0.0456
Standard Error of Ln*=	0.0330
*t**=	1.38
*p**=	0.1896

NOT a significant difference

* - Data generated from the Natural Log transformed data. This transformation is necessary due to the large range the data. It reduces the chances for error.

Patient Sample Comparison

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)	
1	196	197	170	195	163	renal failure
2	17	8	15	20	14	kidney stones
3	920	943	580	568	478	renal failure/hyperPTH
4	176	144	161	220	148	n/a
5	17	11	12	15	15	renal failure
6	375	540	366	542	341	renal failure
7	66	75	51	60	60	renal failure
8	28	25	26	26		renal failure
9	50	59	35	34	38	renal failure
10	89	104	80	79	75	renal failure
11	1043	1160	825	988	831	renal failure
12		0	6			renal failure
13	166	209	126	126	117	bone disease
14	78	99	69	115	68	renal failure
15	31	35	28	32	31	renal failure
16	17	11	17			kidney stones
17	930	959	552	588	473	renal failure/hyperPTH
18	202	192	166	174	172	renal failure
20	143	159	0	141	92	renal failure
21	121	120	69	63	72	renal failure
22	773	850	498	604	523	renal failure/hyperPTH
23	34	27	24	28	23	renal failure
24	104	105	84	85	79	renal failure
25	11	7	12	12	11	renal failure
26	293	316	211	194	186	renal failure
27	105	135	87	81	86	renal failure
28	473	495	353	380	370	renal failure
29	28	22		27	24	n/a
30	74	94	71	70	62	renal failure
31	69	88	59	48	55	renal failure
32	892	1000	677	787	651	renal failure/hyperPTH
33	14	6	13		18	renal failure
34	648	629	575	526	544	renal failure
35	2043	2476	1942	2230	1904	renal failure/hyperPTH
46	1142	1233	725	860	680	renal failure/hyperPTH
47	31	29	31		31	kidney stones
48	877	983	593	613	533	renal failure/hyperPTH
49	143	143	107	102	104	renal failure
50	49	42	33	31	35	renal failure

Significantly Different Groups $p<$ 0.0001

There are two sharp, well separated groups, by Tukey's test

Group 1
Nichols
DSL

Group 2
INCSTAR
EXP(1-7)
EXP(7-84)

CONCLUSIONS

In the Nichols Allegro™ Intact PTH, DSL Active™ PTH and INCSTAR N-tact® PTHSP equivalence of values for samples with clearly defined 1-84 was validated using spikes throughout the standard range, and dilutions to verify values. There is no significant difference for PTH(1-84) values between the three kits ($p = 0.45$). Values for renal samples show a significant difference between the INCSTAR Assay and the other two kits (Nichols and DSL) which report up to twice the INCSTAR value ($p < 0.0001$). PTH (7-84) immunoreactivity was the same for the Nichols, DSL, and one experimental INCSTAR tracer. Two differently purified tracers from INCSTAR with significantly different PTH(7-84) immunoreactivity did not report different values for renal samples, so PTH(7-84) immunoreactivity itself does not elucidate non-(1-84) PTH immunoreactivity. These results suggest that the differences between INCSTAR assay values and the other two kits (Nichols and DSL) is the differing levels of cross-reactivity to fragments present in renal samples, likely of parathyroid gland origin. Based on specificities of each tracer, the N-termini of the fragment(s) reside inside the PTH (7-28) region, and likely end in the PTH (13-28) region. If these data are confirmed in sequencing the fragment, it would not have PTH-receptor mediated biological activity.

Materials and Methods

Assaying of Spiked samples and patient samples:

Respective assay protocols were followed for the collection of data. The PTH(1-84) and PTH(7-84) were purchased from BACHEM. The serum matrix used to spike into was low pH stripped, charcoal stripped, defibrinated, delipidated normal serum. The peptides were dissolved in 5% acetic acid, and serially diluted in the serum matrix to target values within each assays standard range. The spikes were targeted to values based on weight on the vial label, and the entire contents were dissolved with no quantitative assessment of a concentration. All samples were assayed side by side in each kit. The neat value for the serum matrix without spiking was 0.0 pg/mL in both assays. Both Nichols and INCSTAR kit controls were assayed in each others kits and reported as (1-84) spikes.

Peptide Coated Wells for the Specificity Screen:

(modified from Ball et. al., J. Imm. Meth. 171(1994) 37-44)
Briefly: Poly Lys:Tyr (1:1) is coated in PBS to microtiter plates, washed, then the Lys amines are activated with glutaraldehyde. The plates are washed again, and peptides are added to specific wells and left to conjugate over night. The Shiff bases are reduced to primary amines with sodium cyanoborohydride, then the wells are blocked to prevent NSB.

PATENT
Docket No. 532212801100

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Marian Christopher

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the Reexamination of:

Cantor, et al.

Patent No.: 6,689,566 B1

Issue Date: February 10, 2004

Assignee: Scantibodies Laboratory, Inc.

Examiner: To be assigned

DECLARATION OF JOHN COLFORD

MS Ex Parte Reexam
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, John Colford, declare as follows:

1. I am one of the listed co-authors of following documents:
   - Colford J, Salvati M, MacFarlane G, Sokoll L, and Levine M. (1997), entitled "Isolation and Characterization of Large Molecular Weight Fragments of PTH," #P3-194 79th Annual Meeting of the Endocrine Society Program and Abstracts, Minneapolis, MN, U.S.A. (Colford 1997 Abstract) (Ex. 1);
   - Presentation material of Colford 1997 Abstract (Colford 1997 Presentation) (Ex. 2); and

The poster from the 1996 Annual Meeting of the Endocrine Society, San Francisco, CA, U.S.A., Todd Jensen, Jon Spring, and John Colford, entitled "COMPARING SPECIFICITY FOR INTACT HUMAN PARATHYROID HORMONE BETWEEN INCSTAR PTH SP AND NICHOLS INTACT PTH ASSAYS" (Jensen 1996 Poster) (Ex. 3).

2. I have personal knowledge of the subject matters described in the above documents.

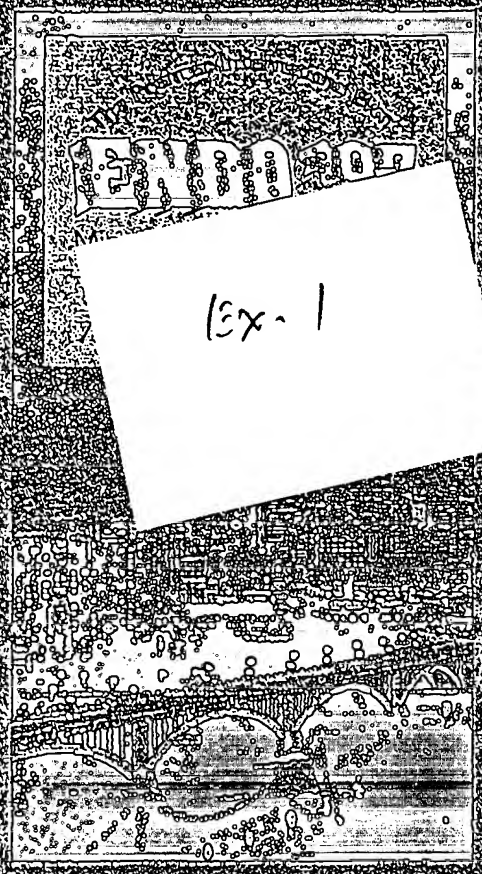
3. Both the Colford 1997 Abstract and the Colford 1997 Presentation refer to a "PTH (1-7) antibody." (Ex. 1 at page IMU-3281 and Ex. 2 at pages IMU-3283, IMU-3284 and IMU-3288.) Jensen 1996 Poster refers to "EXP (1-7)." (Ex. 3 at pages 4, 5 and 9.) The "EXP (1-7)" in the Jensen 1996 Poster refers to an immunoassay for PTH in which the "PTH (1-7) antibody" referred to in the Colford 1997 Abstract and the Colford 1997 Presentation was used.

I declare under penalty of perjury of the laws of the United States that the foregoing is true and correct and that this declaration was executed at HAMPTON, Minnesota on August 19, 2005.

John Colford

John Colford

PROGRAM & ABSTRACTS



79th ANNUAL MEETING

JUNE 11-14, 1997

MINNEAPOLIS, MINNESOTA

THE GEOLOGICAL SOCIETY

Ex. 1

EXHIBIT 1

P03-194
Friday June 13 , 1997
PTH and Calcium [BASIC - Poster Session] (Exhibit Hall 1&2)

Isolation and characterization of large molecular weight fragments of PTH

J.W. Colford[1], M. Salvati[1], G. MacFarlane[1], L.J. Sokoll[2], M.A. Levine[2]

[1] *INCSTAR Corp., Stillwater, MN, USA*
[2] *The Johns Hopkins School of Medicine, Baltimore, MD. USA*

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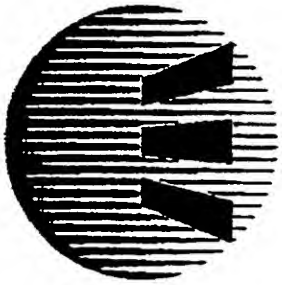
Biochemistry: other
Parathyroid hormone



THE ENDOCRINE SOCIETY

Program & Abstracts

Ex. 2

79th Annual Meeting

June 11-14, 1997 Minneapolis, Minnesota

EXHIBIT 2

IMU-3280

P3-194

ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH. JW Colford-[1], M Salvati[1], G MacFarlane[1], LJ Sokoll[2], and MA Levine[2]. [1]INCSTAR Corp., Stillwater, MN 55082; [2]The Johns Hopkins School of Medicine, Baltimore, MD 21205.

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

JWC

#P3-194: ISOLATION AND CHARACTERIZATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF PTH.

JW Colford*[1], M Salvati[1], G MacFarlane[1], LJ Sokoll[2], and MA Levine[2]. [1]INCSTAR Corp., Stillwater, MN 55082; [2]The Johns Hopkins Medical Institutions, Baltimore, MD 21205.

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Introduction

Intact PTH secretion is equisitely controlled by serum ionized calcium in a classic negative feedback loop. Sensitive calcium receptors on parathyroid chief cells detect very slight changes in the concentration of Ca^{2+} and secrete intact PTH in response to low concentrations. Total serum calcium exists in three phases in dynamic equilibrium: protein bound (mainly albumin, Ig), complexed (phosphate, bicarbonate, citrate), and ionized calcium.

The clinical utility of a PTH measurement, in combination with a serum calcium determination, is in diagnosis of primary hyperparathyroidism, management of uremic or seconday hyperparathyroidism, and diagnosis of hypoparathyroidism.

Previously characterized serum PTH heterogeneity includes the intact PTH (1-84) active peptide hormone, and inactive C-terminal fragments with N-termini between PTH (34-43). Thus, strategies for immunoassay evaluation of intact PTH concentrations in serum involved a solid phase antibody present in excess to overcome interfering C-terminal fragments and thus detecting intact PTH only using a N-terminally directed reporter antibody.

N-Terminal truncations yielding PTH species larger than PTH 34-84 have been isolated from human parathyroid cell monolayer.C-terminal fragments have been detected in extracts of parathyroid gland, and parathyroid effluent samples collected from hypercalcemic patients with hyperparathyroidism. Based on immunoassay value differences observed by our group and PTH immunoreactivity by Brossard et.al. (JCEM 81(11): 3923-3929) there appears to be differences in immunoassay values caused by fragment recognition in some commercially available intact PTH assays.

Evidence for a circulating inhibitor to PTH has arisen from studies of patients with pseudohyperparathyroidism. Despite elevated levels of immunoreactive PTH, bioactive PTH was normal when tested in bioassay systems. Plasma from these patients has been shown to diminish the biological activity of exogenous PTH in these *in vitro* bioassays.

To determine what PTH molecular forms circulate, and elucidate possible biological activity, a detection system was developed to elucidate the concentrations of intact PTH and the novel large molecular weight fragments of PTH, without interference or recognition of the hepatically generated C-terminal fragments. Data generated was evaluated for diagnostic potential with regard to primary hyperparathyroidism, and correlation to serum calcium in primary and uremic hyperparathyroidism.

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Abstract

Differences exist among commercial assay values for intact PTH. In comparing the Nichols Allegro™ Intact PTH assay to the INCSTAR N-tact® PTH SP Intact PTH kit, we found that samples from patients with uremic or primary hyperparathyroidism yielded values 1.4 times higher, and 1.2 times higher, respectively, in the Nichols assay than the INCSTAR assay. Both the assays use the same C-terminal PTH (39-84) capture antibody, but each assay has its own N-terminal tracer antibody. To evaluate whether truncated forms of PTH may account for these differences, antibody directed to N-terminal PTH (1-7) was adsorbed on beads and intact PTH was immunoextracted from pooled sera from patients with uremic or primary hyperparathyroidism. Despite exhaustive immunoextraction, 68% of the assay value difference persisted between the INCSTAR and Nichols intact PTH values. A second antibody directed to PTH (13-34) was adsorbed on beads and this solid phase eliminated 96% of immunoreactivity of both kits. These data suggested the presence of an N-terminally truncated fragment of PTH that was larger than had been previously characterized. To isolate the non-(1-84) molecular form(s), an immunoextraction resin was made by conjugating an antibody specific for PTH (39-84) to hydrophobic macroporous polymeric beads. Delipidated pooled uremic serum was passed over the column, the column was washed with 0.5M NaCl, and bound protein was eluted with low pH glycine buffer. The protein in the eluent was separated by reverse phase HPLC and the fractions were dried with N_2 and reconstituted in stripped serum. These samples were tested using the Nichols assay; plotting the sample values by retention time showed two major peaks with a shoulder on the first peak, suggesting one or two immunoreactive PTH species in addition to intact PTH. By contrast, an assay using PTH (1-7) antibody as the tracer showed only one major peak. We conclude that PTH heterogeneity in the circulation of patients with renal failure and primary hyperparathyroidism is more extensive than previously thought. The fragment(s) are truncated at the N-terminus in the (7-28) region and are detected in some commercially available assays for intact PTH. It has been shown that PTH (1-14) appears necessary for receptor activation upon receptor binding, while PTH (15-34) provides most of the binding affinity, and thus the fragment(s) is not a PTH/PTHrP receptor agonist, but remains a candidate as a receptor antagonist.

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PTH Values inUremic Hyperparathyroidism

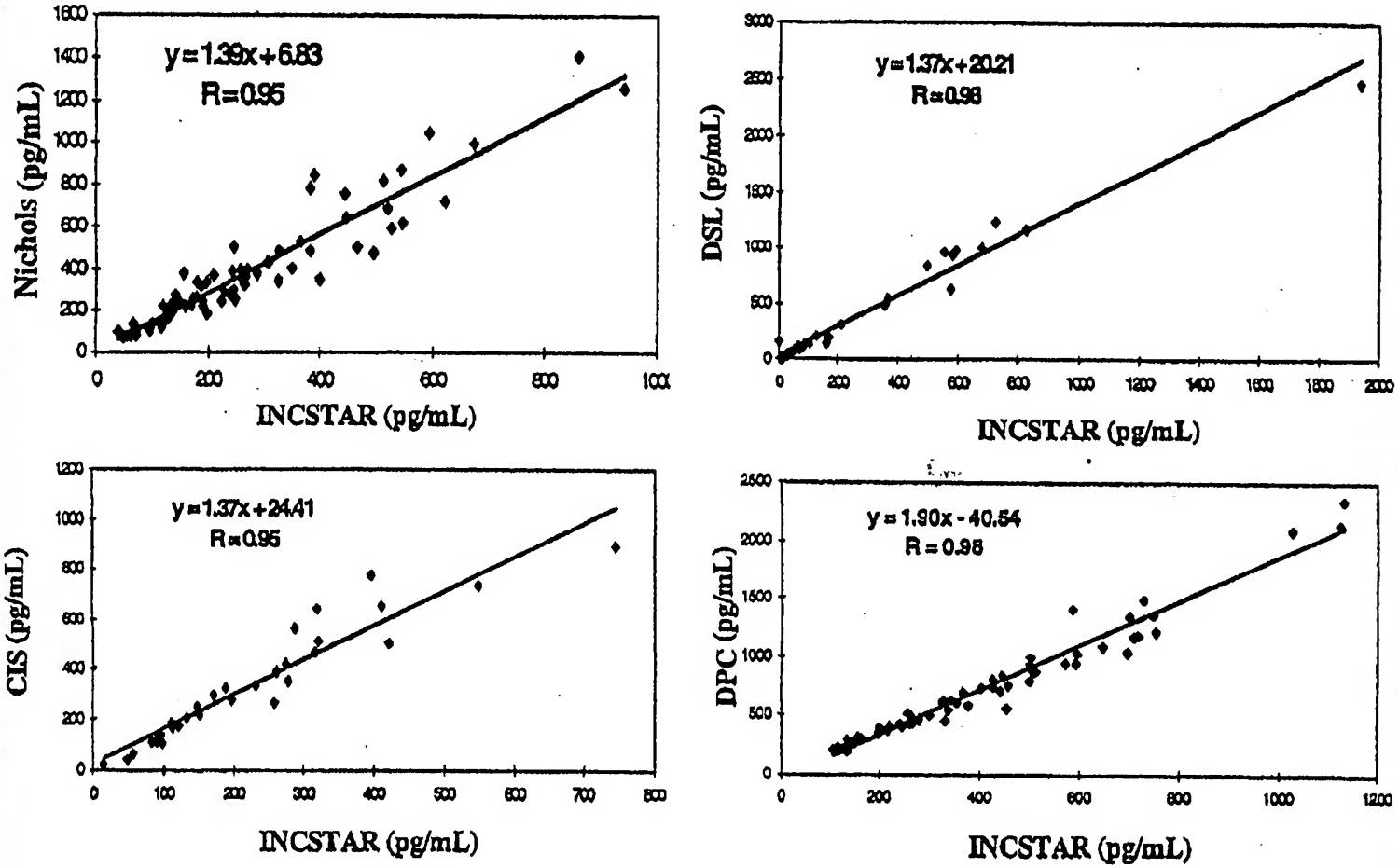

Figure 1. Correlations of several commercially available intact PTH assays verses the INCSTAR N-tact PTH SP intact PTH assay. All of the commercial assays evaluated indicate much greater cross-reactivity to the novel PTH fragments.

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INCSTAR PTH Values vs. Nichols PTH Values

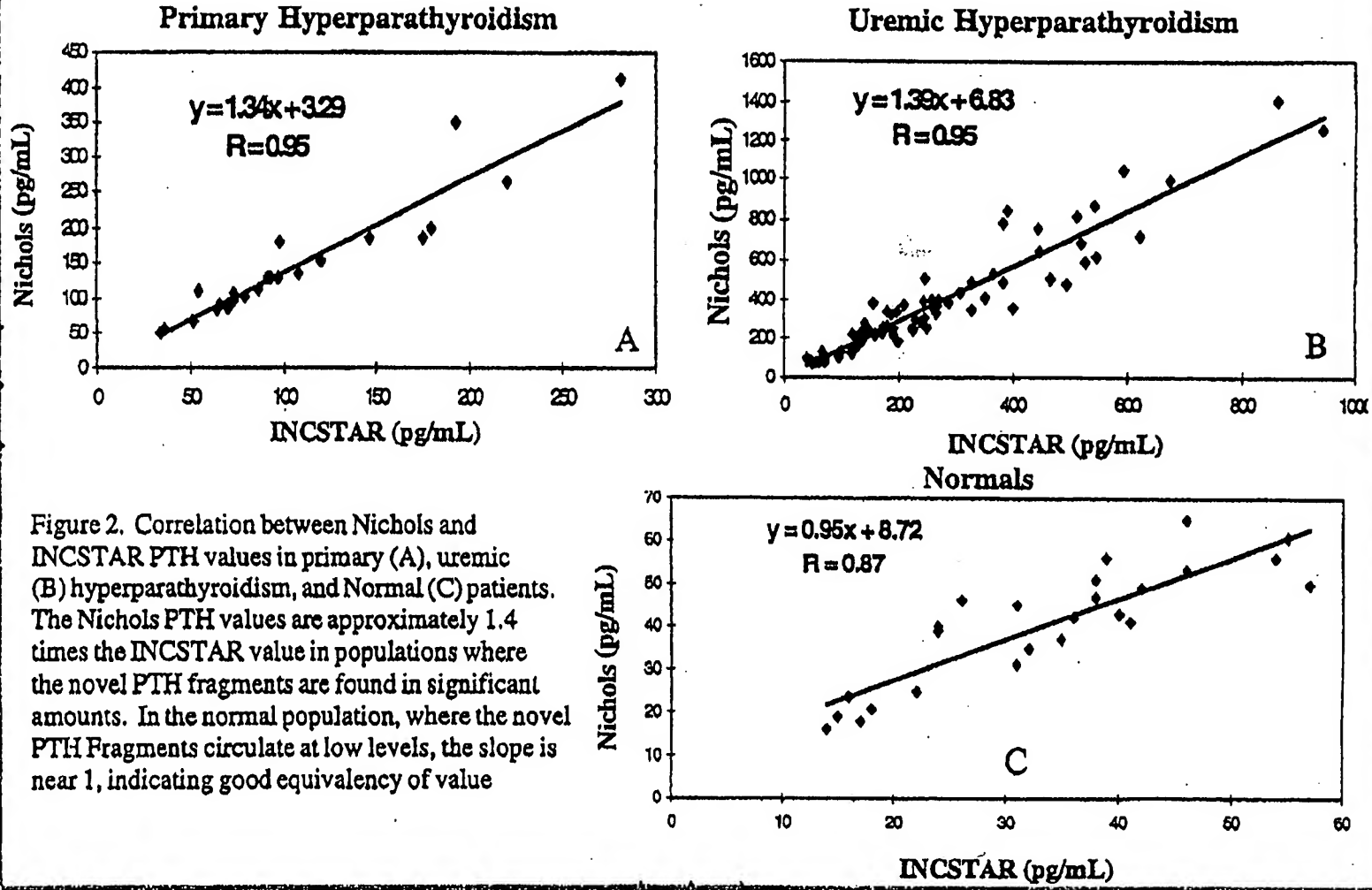

Figure 2. Correlation between Nichols and INCSTAR PTH values in primary (A), uremic (B) hyperparathyroidism, and Normal (C) patients. The Nichols PTH values are approximately 1.4 times the INCSTAR value in populations where the novel PTH fragments are found in significant amounts. In the normal population, where the novel PTH Fragments circulate at low levels, the slope is near 1, indicating good equivalency of value

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Method of Isolation of PTH Molecular Forms

Delipidated EDTA plasma is loaded on the column. PTH Molecular forms containing all or parts of the (39-84) region are captured. The specifically bound protein is eluted with 0.2 M glycine pH 2.5

Anti-PTH(39-84) Immunoextraction Column

The protein eluted from the immunoextraction step is loaded onto a C_{18} reverse-phase HPLC column. The column resolves homologous proteins by size. 2-60% - 0.1% TFA/Acetonitrile : 0.1% TFA/dH20 over 58 minutes 1%/minute.

C_{18} Reverse Phase HPLC Column

HPLC Fractions were tested for PTH immunoreactivity

Figure 3. Method of isolation of PTH molecular forms.

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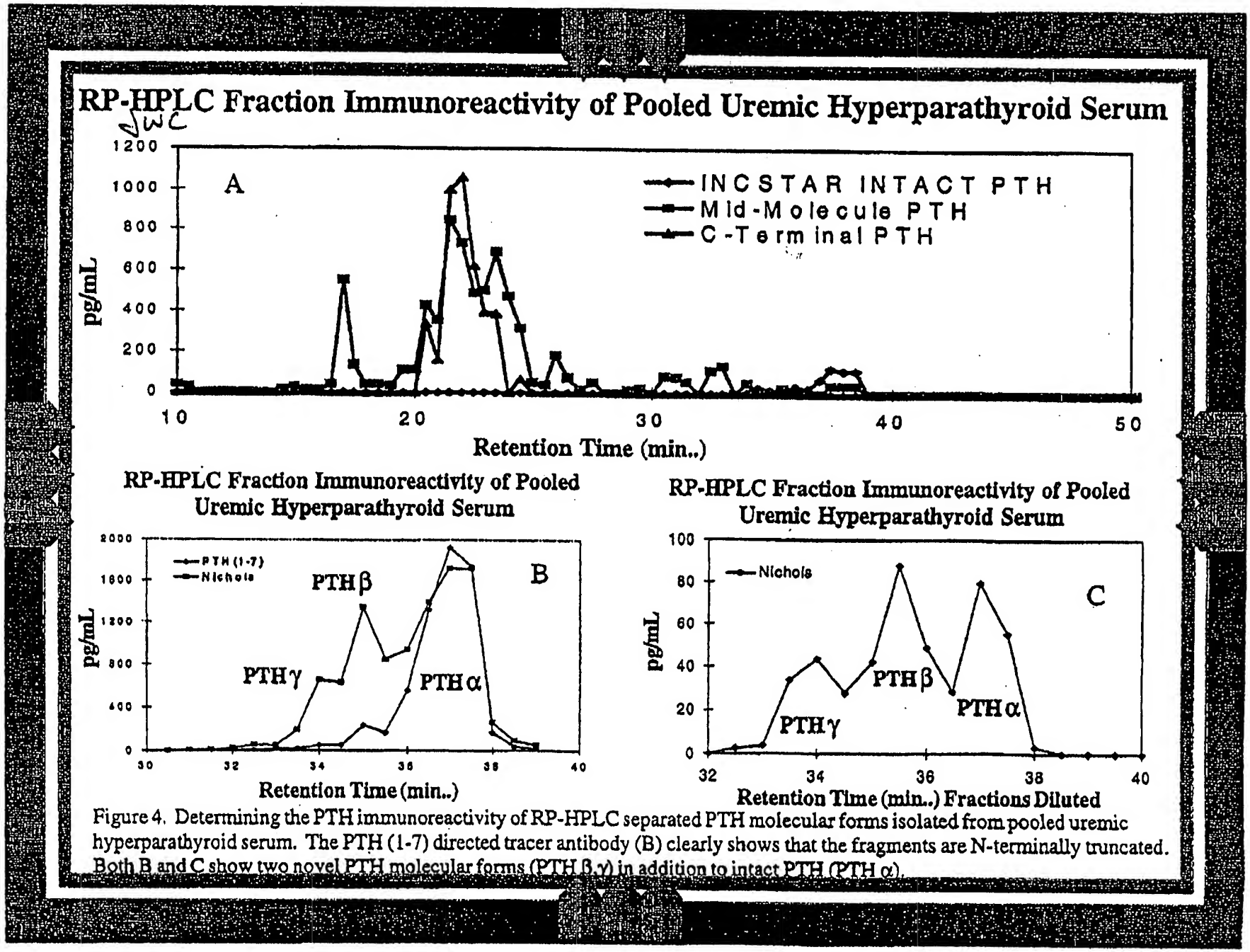

Figure 4. Determining the PTH immunoreactivity of RP-HPLC separated PTH molecular forms isolated from pooled uremic hyperparathyroid serum. The PTH (1-7) directed tracer antibody (B) clearly shows that the fragments are N-terminally truncated. Both B and C show two novel PTH molecular forms (PTH β,γ) in addition to intact PTH (PTH α).

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PTH Molecular Forms

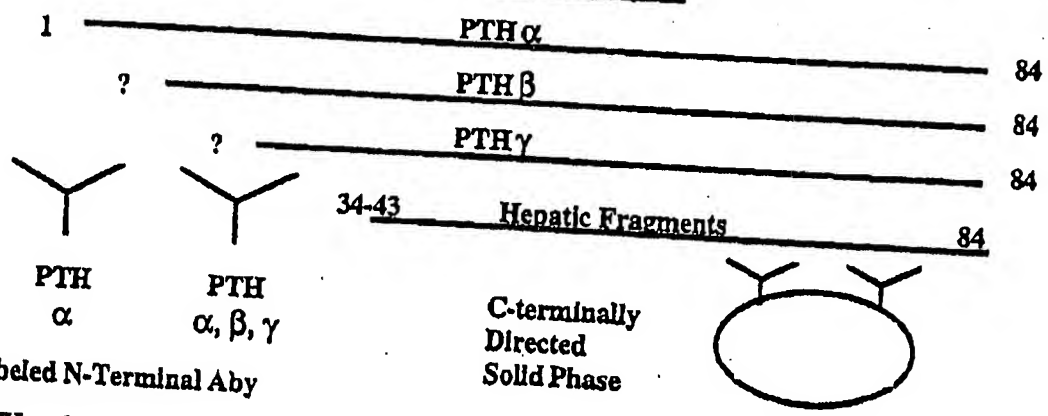

Figure 5. Defining PTH molecular forms isolated by RP-HPLC and the detection system used to estimate their concentration. These fragments have N-termini that extend beyond amino acid 34, and do not include hepatically generated fragments.

PTH Ratio

$$\frac{[\text{PTH } \alpha, \beta, \gamma] - [\text{PTH } \alpha]}{[\text{PTH } \alpha]} = \frac{\text{PTH } \beta,\gamma}{\text{PTH } \alpha}$$

This calculation yields a ratio of the two novel C-terminal Fragments / Intact PTH

The hepatically generated C-terminal fragments are NOT evaluated in this system

Figure 6. Defining the PTH Ratio to be the concentrations of the two novel PTH fragments to the concentration of intact PTH

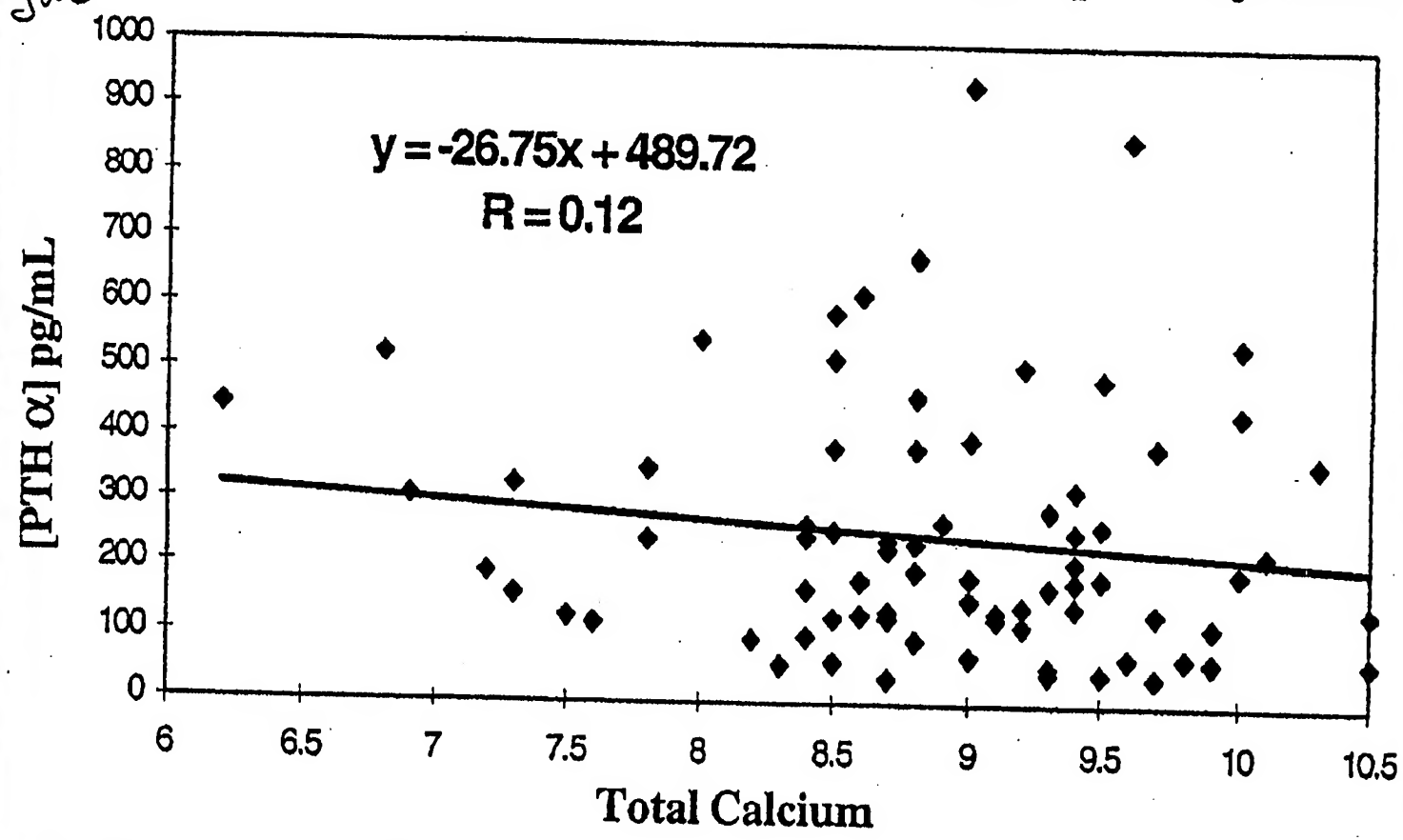

Figure 7. This figure plotted intact PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernable relationship between total calcium and total active PTH in this uremic hyperparathyroid population.

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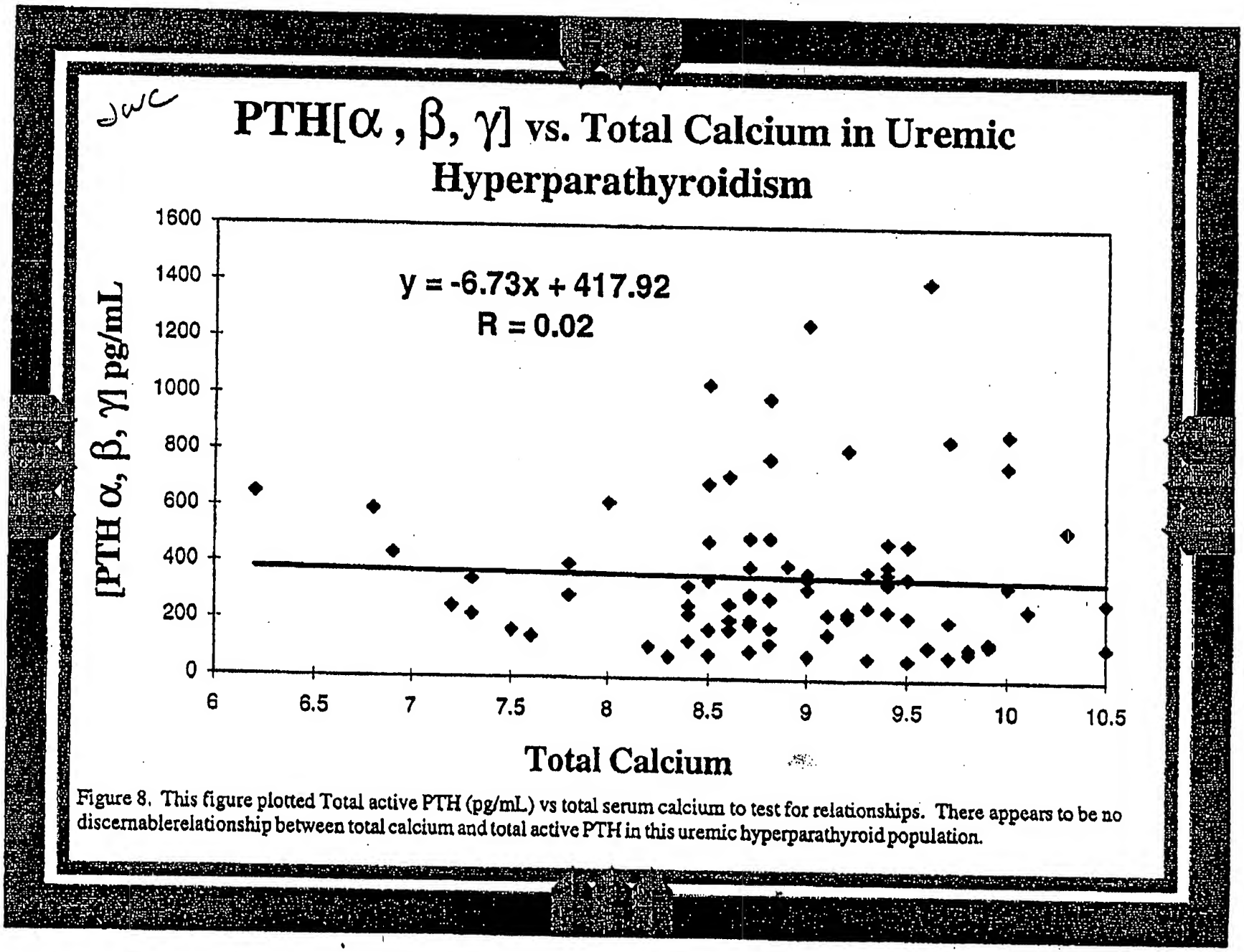

Figure 8. This figure plotted Total active PTH (pg/mL) vs total serum calcium to test for relationships. There appears to be no discernablerelationship between total calcium and total active PTH in this uremic hyperparathyroid population.

PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Uremic Hyperparathyroidism

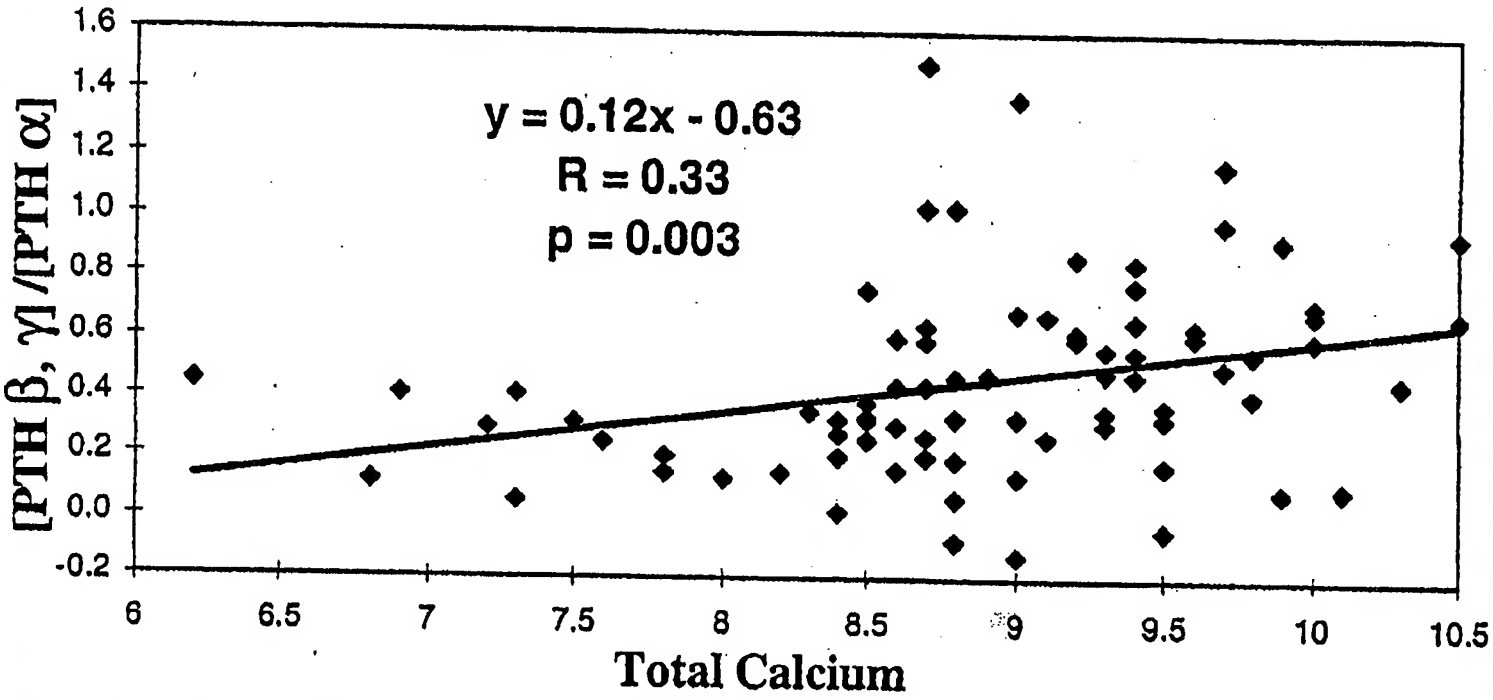

Figure 9. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, the corresponding serum calcium approaches normalization in uremic hyperparathyroidism even though most of these samples have elevated intact PTH levels. This trend is opposite to that in primary hyperparathyroidism. Endogenous and exogenous factors, such as calcitriol therapy, complicate calcium homeostasis in uremic hyperparathyroidism, and thus cause and effect conclusions cannot be made in this population without more information. However, this is consistent with an inhibitory effect of these fragments to PTH in these patients.

JWC PTH[β, γ]/ [PTH α] Ratio vs. Total Calcium in Primary Hyperparathyroidism

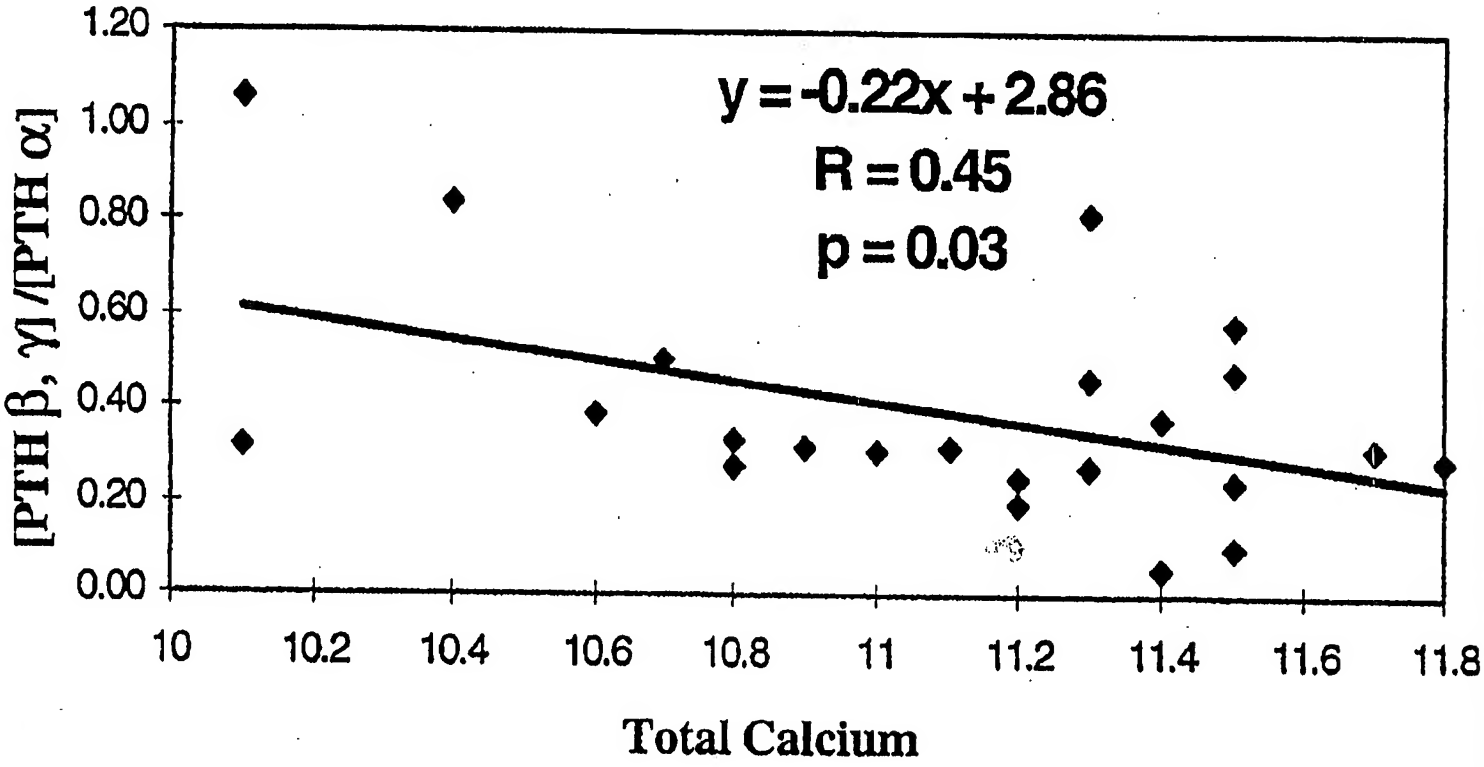

Figure 10. This figure plotted PTH Ratio vs total serum calcium to test for relationships. As the concentration of the fragments approach that of intact PTH, and the ratio approaches 1, there is a trend of normalization of serum calcium in primary hyperparathyroidism even though all of these samples have elevated intact PTH levels. This is consistant with an inhibitory effect of these fragments to PTH action in this population.

IMU-3293

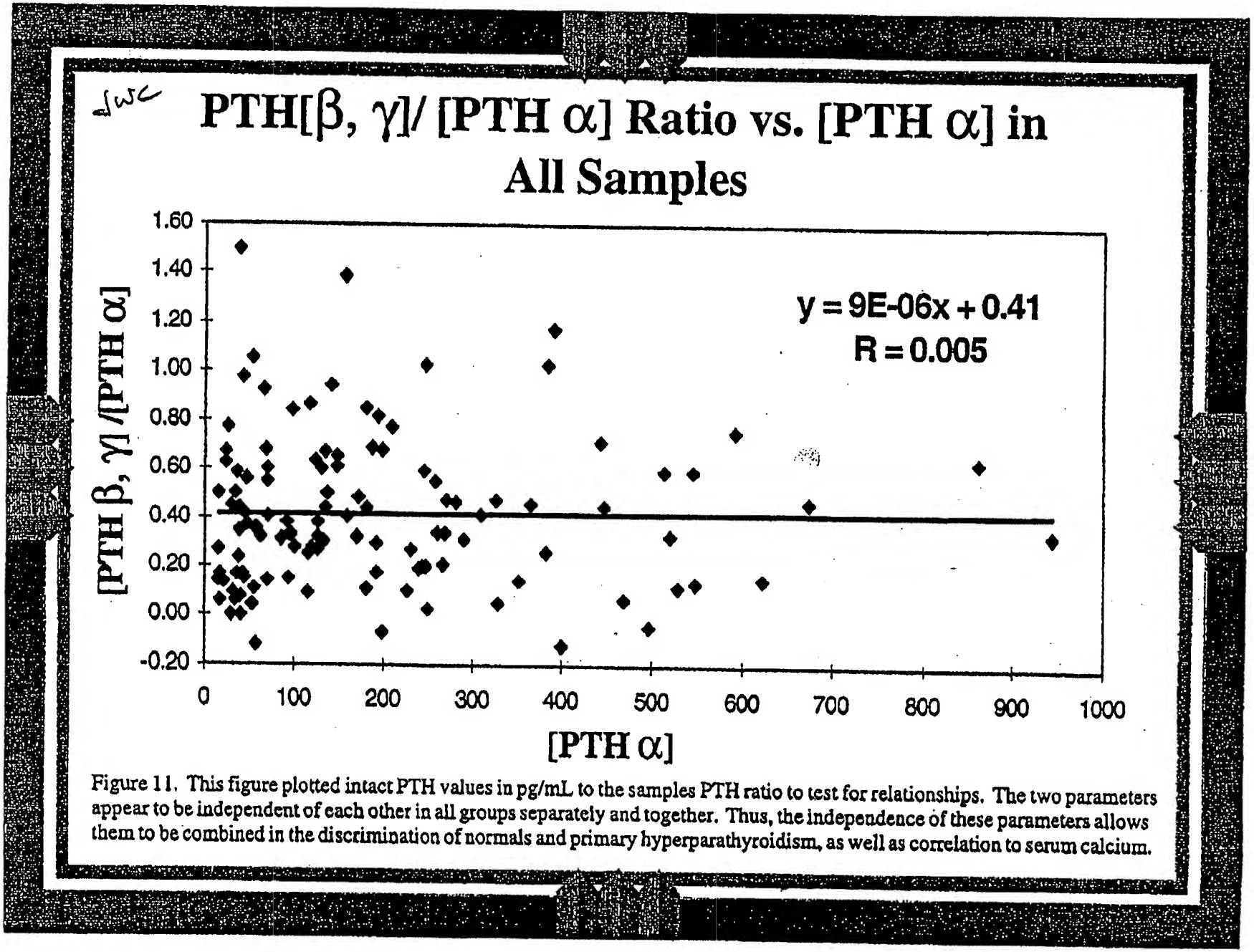

Figure 11. This figure plotted intact PTH values in pg/mL to the samples PTH ratio to test for relationships. The two parameters appear to be independent of each other in all groups separately and together. Thus, the independence of these parameters allows them to be combined in the discrimination of normals and primary hyperparathyroidism, as well as correlation to serum calcium.

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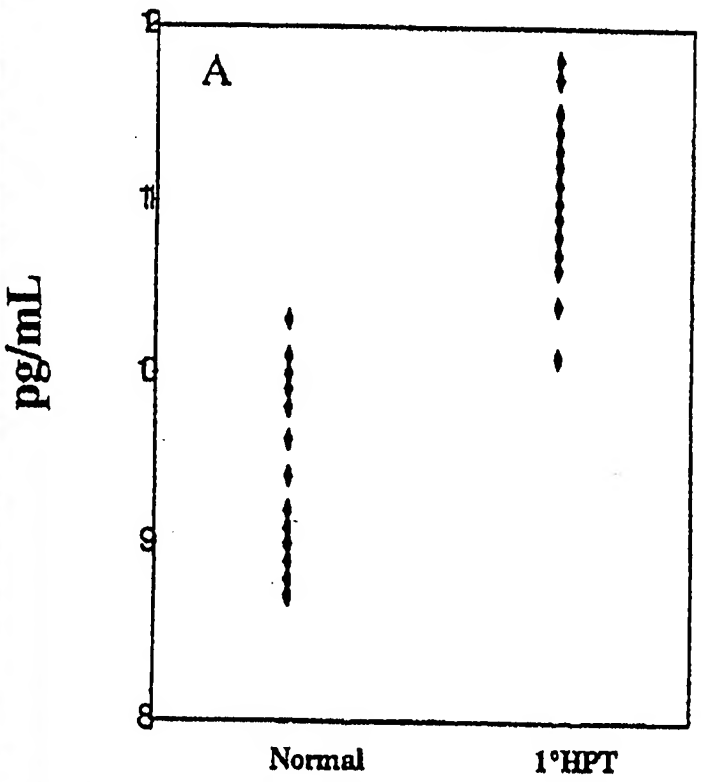
Discrimination Power of Total Calcium



Discrimination Power of PTH

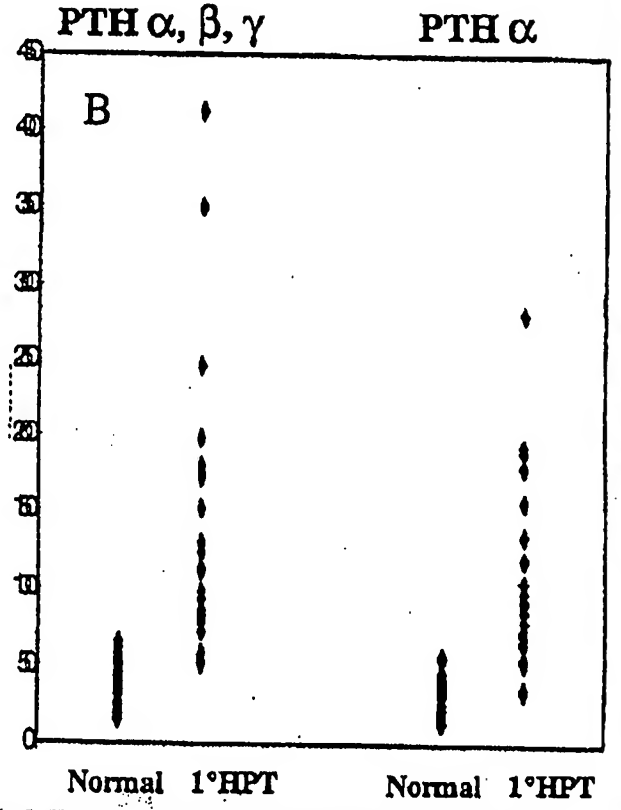


Figure 12. This figure used serum total calcium (A) and PTH values in pg/mL(B) to compare the discrimination power of calcium and PTH measurement in the separation of a normal and primary hyperparathyroid population. There is clearly significan overlap of the populations using both calcium and PTH determinations separately. These parameters are typically combined in a clinical situation to provide optimal discrimination, but which PTH parameter is the best is evaluated in Figure 13.

Discrimination Power of PTH measurements + Total Calcium

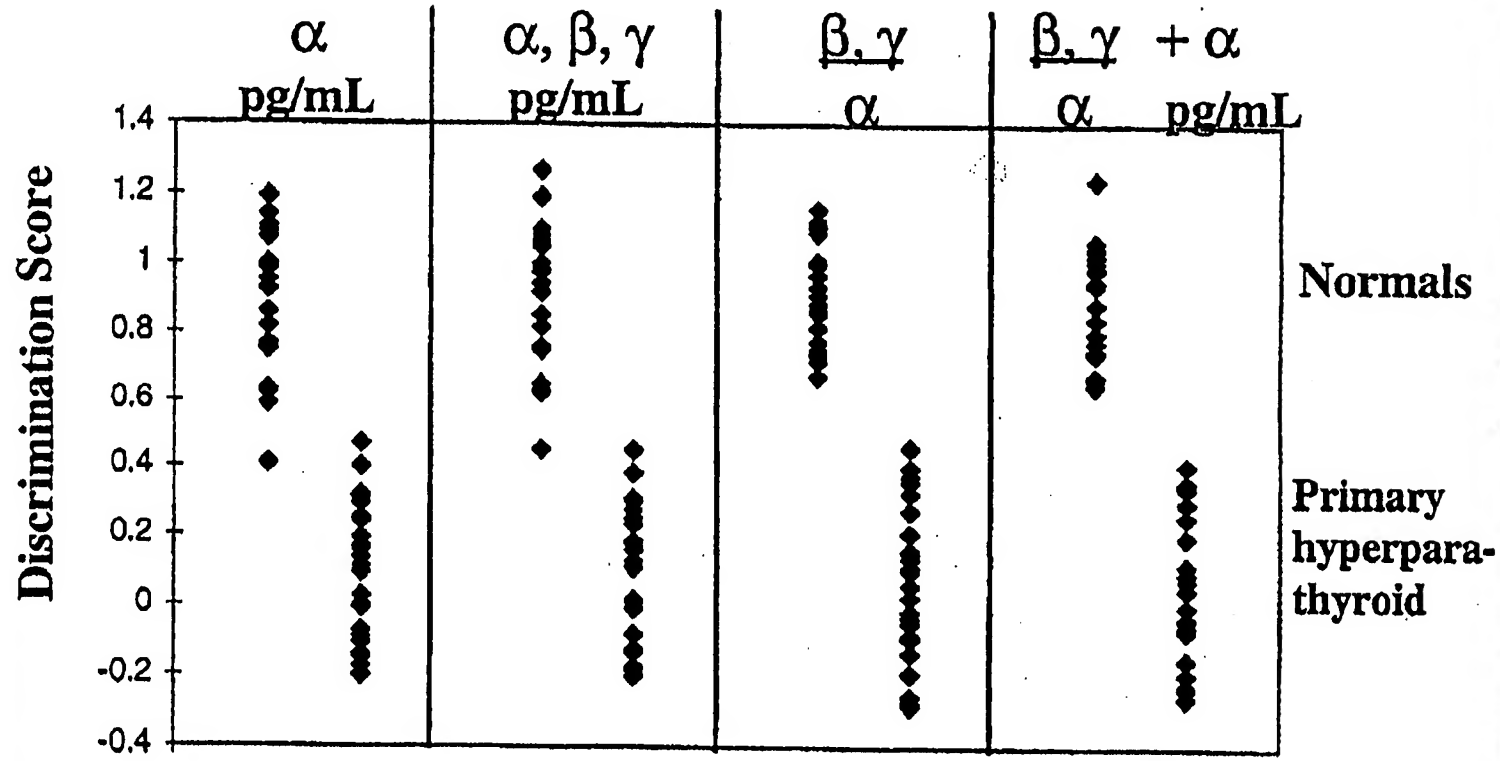


Figure 13. This figure used regression analysis to compare the discrimination power of a PTH measurement in addition to a total calcium determination in the separation of a normal and primary hyperparathyroid population. The combination of a PTH measurement with a serum calcium determination is the common clinical situation in diagnosis of primary hyperparathyroidism. The PTH ratio allows clear distinction of borderline cases into the respective populations.

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CONCLUSIONS

- There appears to be PTH heterogeneity found in circulation in patients with primary and uremic hyperparathyroidism beyond what has been characterized thus far.
- These novel PTH fragments appear to be N-terminally truncated and are reported in some commercially available intact PTH assays.
- As levels of the fragments approach those of Intact PTH, serum calcium trends toward normalization in patients with primary and uremic hyperparathyroidism
- Based on the data and references, we speculate that these fragments are a strong candidate for being a competitive inhibitor to intact parathyroid hormone action at the PTH/PTHrP receptor in patients with primary hyperparathyroidismand mediate its biological activity.
- Complete characterization of PTH molecular forms provides a better correlation to serum calcium, and provides greater clinical discrimination between primary hyperparathyroid and normals.

COMPARING SPECIFICITY FOR INTACT HUMAN PARATHYROID HORMONE BETWEEN INCSTAR PTHSP AND NICHOLS INTACT PTH ASSAYS.

Todd Jensen, Jon Spring, and John Colford (INCSTAR Corporation, Stillwater, MN 55082)

EXHIBIT 3

ABSTRACT

The N-terminus of the PTH molecule is required for PTH receptor interaction. It has been shown that cleavage of three amino acids from the N-terminus will inactivate PTH activity *in vivo*. Specificity is an advantage of a two antibody assay format of an IRMA. An N-terminal truncated circulating inhibitor to PTH has been hypothesized, but not found. Both the INCSTAR Intact PTHSP™ assay and the Nichols Allegro™ Intact PTH claim to be specific for the intact molecule. In a comparison between the two kits, patients with renal failure were assayed. The results showed that the Nichols assay results to be an average of 1.52 times the INCSTAR assay value (n = 14). Intact 1-84 PTH was spiked into clarified human serum and assayed in both kits. The results showed that the Nichols assay results to be an average of 1.07 times the INCSTAR assay value (n = 12).

Both assay tracers are directed to the N-terminal region of the intact PTH molecule. To compare specificity of the two assay tracers, we used a (7-84) C-terminal fragment. While not a defined byproduct of PTH metabolism *in vivo*, it could provide insight to the degree the population of antibodies in the tracer are directed to the N-terminus. Based on weight, target levels of 1500, 1000, and 500 pg/ml (7-84) PTH C-terminal fragment were spiked into clarified human serum. the Nichols assay had values of 520, 374, and 245 pg/ml, respectively. The INCSTAR assay had values of 220, 167, and 114 pg/ml, respectively. This showed that the Nichols results to be an average of 2.25 times the INCSTAR assay value (n = 14).

The INCSTAR tracer was substituted into the Nichols assay and the (7-84) PTH spiked serum samples were assayed, with the hybrid assay reporting 258, 191, and 121 pg/ml, respectively. This suggests that the INCSTAR tracer gives the INCSTAR assay greater specificity for the intact PTH molecule than the Nichols assay. These results also suggest the presence of a C-terminal fragment of PTH in renal samples that has not been currently identified.

INTRODUCTION

Recently, it has been reported that there is a non-(1-84) molecular form of PTH found in normals and renal samples that is immunoreactive in the Nichols Allegro™ Intact PTH assay (Brossard et al, Proc. Int. Cong. Endo, Vol2, OR60-5). The PTH molecule is degraded in the parathyroid gland itself and also in the liver. The breakdown products don't have most of the N-terminal 27 amino acids and lack hypercalcemic, hypocalciuric, and phosphaturic activity. These fragments have a considerable half-life and accumulate in circulation up to 20 times the concentration of the intact hormone. Their presence makes the immunologic assessment of the biologically active hormone difficult.

The hepatic fragments are generated when enzymes on the surface of Kupffer cells cleave the hormone in its 34-43 region. These fragments are formed at a rate dependent largely on the concentration of intact PTH. The N-terminal fragment is at very low concentrations in serum, suggesting a quick and thorough removal from circulation. Fragments generated in the parathyroid gland are truncated at both ends of the molecule. These fragments are not currently defined. In hypercalcemic patients of a non-parathyroid origin, the negative feedback mechanism will suppress PTH release to almost nothing, but the parathyroid gland will continue to release truncated fragments. In patients that have nonparathyroid forms of hypercalcemia, these fragments have been detected by a carboxy- terminal RIA but the N-terminus was not defined. All PTH fragments are removed from circulation by the kidney. In cases of renal insufficiency, fragments accumulate.

There appear to be differences in the values for renal samples among the Nichols Allegro™, DSL Active™, and INCSTAR N-tact® PTHSP assays. To explore these differences, PTH (1-84) and (7-84) immunoreactivity was validated between the assays. If PTH (1-84) equivalence is shown, renal sample differences will be significant. The results will be compared to reported non-(1-84) PTH immunoreactivity.

Dilution Linearity Validation

				% Recovery	
Pat. ID#	**46**	**46 1:2**	**46 1:4**	**46 1:2**	**46 1:4**
Nichols	1142	573	277	100	97
DSL	1233	707	378	115	123
INCSTAR	725	354	159	98	88
EXP(1-7)	860	383	158	89	74
EXP(7-84)	680	343	158	101	93

			% Recovery
Pat. ID#	**48**	**48 1:2**	**48 1:2**
Nichols	877	485	111
DSL	983	546	111
INCSTAR	593	278	94
EXP(1-7)	613	300	98
EXP(7-84)	533	253	95

			% Recovery
Pat. ID#	**49**	**49 1:2**	**49 1:2**
Nichols	143	67	94
DSL	143	69	96
INCSTAR	107	51	95
EXP(1-7)	102	44	87
EXP(7-84)	104	59	112

PTH(7-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(7-84)
1	272	217	193	238
2	515	395	296	393
3	1262	1168	816	1094
4	1720	1623	1059	1693

$p<$ 0.0004

INCSTAR Tracer is unique
Nichols, DSL, and EXP (7-84)
are not shown different

PTH(1-84) Spikes into Patient Samples

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)
1	175	168	175	201	182
2	188	184	222	263	218
3	696	772	620	648	615
4	1058	1152	1045	1074	988

$p=$ 0.45

No Observed Difference

PTH Tracer Specificity.

	NSB	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	20	5419	40	1452	3643	3294	8	0	12	14	19
DSL	559	11627	415	5585	8922	6916	556	364	424	319	278
Nichols	195	2982	139	968	2606	2136	153	138	139	220	142

Yes is a signal 2 SD above Background

	1-84	1-7	1-34	7-84	13-34	28-48	44-68	44-86	53-84	70-84
INCSTAR	Yes	Yes	Yes	Yes	Yes	No	No	No	No	No
DSL	Yes	No	Yes	Yes	Yes	No	No	No	No	No
Nichols	Yes	No	Yes	Yes	Yes	No	No	No	No	No

Values from two separate assays confirming differences in (7-84) Spiked serum values between Nichols and INCSTAR

FRAGMENT(7-84) SPIKES (pg/mL)

	Target Value*	INCSTAR Assay	Nichols Assay	DIFFERENCE
Assay 1	1500	511	774	-262
	1000	366	577	-211
	500	220	346	-125
Assay 2	1000	220	520	-300
	400	167	374	-207
	250	114	245	-131

* - Value based on assumption of 100% purity and quantitative transfer in dilution

MEAN=	-206.0	
Variance=	4835.4	
Standard Error=	28.39	
t=	7.26	
p=	0.00078	Significant difference

Comparison of (1-84) Spiked serum values and controls between Nichols and INCSTAR

		INTACT (1-84) (pg/mL)		
		INCSTAR	Nichols	DIFFERENCE
Serum Spikes	spike 1	2805	2883	-77
	spike 2	1617	1712	-95
	spike 1 Diluted 1:10	273	236	37
	spike 2 Diluted 1:10	157	152	5
	spike 3	1656	1589	67
	spike 4	1740	1724	16
	spike 3 Diluted 1:10	183	212	-29
	spike 4 Diluted 1:10	164	190	-26
Kit Controls	NJ	36	31	6
	NK	265	214	51
	(INC)L1	33	34	-1
	(INC)L2	415	330	85
	(INC)L1	49	41	8
	(INC)L2	447	426	21

MEAN of Ln*=	0.0456
Standard Error of Ln*=	0.0330
t*=	1.38
p*=	0.1896

NOT a significant difference

* - Data generated from the Natural Log transformed data. This transformation is necessary due to the large range the data. It reduces the chances for error.

Patient Sample Comparison

	Nichols	DSL	INCSTAR	EXP(1-7)	EXP(7-84)	
1	196	197	170	195	163	renal failure
2	17	8	15	20	14	kidney stones
3	920	943	580	568	478	renal failure/hyperPTH
4	176	144	161	220	148	n/a
5	17	11	12	15	15	renal failure
6	375	540	366	542	341	renal failure
7	66	75	51	60	60	renal failure
8	28	25	26	26		renal failure
9	50	59	35	34	38	renal failure
10	89	104	80	79	75	renal failure
11	1043	1160	825	988	831	renal failure
12		0	6			renal failure
13	166	209	126	126	117	bone disease
14	78	99	69	115	68	renal failure
15	31	35	28	32	31	renal failure
16	17	11	17			kidney stones
17	930	959	552	588	473	renal failure/hyperPTH
18	202	192	166	174	172	renal failure
20	143	159	0	141	92	renal failure
21	121	120	69	63	72	renal failure
22	773	850	498	604	523	renal failure/hyperPTH
23	34	27	24	28	23	renal failure
24	104	105	84	85	79	renal failure
25	11	7	12	12	11	renal failure
26	293	316	211	194	186	renal failure
27	105	135	87	81	86	renal failure
28	473	495	353	380	370	renal failure
29	28	22		27	24	n/a
30	74	94	71	70	62	renal failure
31	69	88	59	48	55	renal failure
32	892	1000	677	787	651	renal failure/hyperPTH
33	14	6	13		18	renal failure
34	648	629	575	526	544	renal failure
35	2043	2476	1942	2230	1904	renal failure/hyperPTH
46	1142	1233	725	860	680	renal failure/hyperPTH
47	31	29	31		31	kidney stones
48	877	983	593	613	533	renal failure/hyperPTH
49	143	143	107	102	104	renal failure
50	49	42	33	31	35	renal failure

Significantly Different Groups $p<$ 0.0001

There are two sharp, well separated groups, by Tukey's test

Group 1
Nichols
DSL

Group 2
INCSTAR
EXP(1-7)
EXP(7-84)

CONCLUSIONS

In the Nichols Allegro™ Intact PTH, DSL Active™ PTH and INCSTAR N-tact® PTHSP equivalence of values for samples with clearly defined 1-84 was validated using spikes throughout the standard range, and dilutions to verify values. There is no significant difference for PTH(1-84) values between the three kits ($p = 0.45$). Values for renal samples show a significant difference between the INCSTAR Assay and the other two kits (Nichols and DSL) which report up to twice the INCSTAR value ($p < 0.0001$). PTH (7-84) immunoreactivity was the same for the Nichols, DSL, and one experimental INCSTAR tracer. Two differently purified tracers from INCSTAR with significantly different PTH(7-84) immunoreactivity did not report different values for renal samples, so PTH(7-84) immunoreactivity itself does not elucidate non-(1-84) PTH immunoreactivity. These results suggest that the differences between INCSTAR assay values and the other two kits (Nichols and DSL) is the differing levels of cross-reactivity to fragments present in renal samples, likely of parathyroid gland origin. Based on specificities of each tracer, the N-termini of the fragment(s) reside inside the PTH (7-28) region, and likely end in the PTH (13-28) region. If these data are confirmed in sequencing the fragment, it would not have PTH-receptor mediated biological activity.

Materials and Methods

Assaying of Spiked samples and patient samples:

Respective assay protocols were followed for the collection of data. The PTH(1-84) and PTH(7-84) were purchased from BACHEM. The serum matrix used to spike into was low pH stripped, charcoal stripped, defibrinated, delipidated normal serum. The peptides were dissolved in 5% acetic acid, and serially diluted in the serum matrix to target values within each assays standard range. The spikes were targeted to values based on weight on the vial label, and the entire contents were dissolved with no quantitative assessment of a concentration. All samples were assayed side by side in each kit. The neat value for the serum matrix without spiking was 0.0 pg/mL in both assays. Both Nichols and INCSTAR kit controls were assayed in each others kits and reported as (1-84) spikes.

Peptide Coated Wells for the Specificity Screen:

(modified from Ball et. al., J. Imm. Meth. 171(1994) 37-44)
Briefly: Poly Lys:Tyr (1:1) is coated in PBS to microtiter plates, washed, then the Lys amines are activated with glutaraldehyde. The plates are washed again, and peptides are added to specific wells and left to conjugate over night. The Shiff bases are reduced to primary amines with sodium cyanoborohydride, then the wells are blocked to prevent NSB.

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Development of a Novel Immunoradiometric Assay Exclusively for Biologically Active Whole Parathyroid Hormone 1-84: Implications for Improvement of Accurate Assessment of Parathyroid Function

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ABSTRACT

We developed a novel immunoradiometric assay (IRMA; whole parathyroid hormone [PTH] IRMA) for PTH, which specifically measures biologically active whole PTH(1-84). The assay is based on a solid phase coated with anti-PTH(39-84) antibody, a tracer of ^{125}I-labeled antibody with a unique specificity to the first N-terminal amino acid of PTH(1-84), and calibrators of diluted synthetic PTH(1-84). In contrast to the Nichols intact PTH IRMA, this new assay does not detect PTH(7-84) fragments and only detects one immunoreactive peak in chromatographically fractionated patient samples. The assay was shown to have an analytical sensitivity of 1.0 pg/ml with a linear measurement range up to 2300 pg/ml. With this assay, we further identified that the previously described non-(1-84)PTH fragments are aminoterminally truncated with similar hydrophobicity as PTH(7-84), and these PTH fragments are present not only in patients with secondary hyperparathyroidism (2°-HPT) of uremia, but also in patients with primary hyperparathyroidism (1°-HPT) and normal persons. The plasma normal range of the whole PTH(1-84) was 7–36 pg/ml (mean ± SD: 22.7 ± 7.2 pg/ml, $n = 135$), whereas over 93.9% (155/165) of patients with 1°-HPT had whole PTH(1-84) values above the normal cut-off. The percentage of biologically active whole PTH(1-84) (pB%) in the pool of total immunoreactive "intact" PTH is higher in the normal population (median: 67.3%; SD: 15.8%; $n = 56$) than in uremic patients (median:53.8%; SD: 15.5%; $n = 318$; $p < 0.001$), although the whole PTH(1-84) values from uremic patients displayed a more significant heterogeneous distribution when compared with that of 1°-HPT patients and normals. Moreover, the pB% displayed a nearly Gaussian distribution pattern from 20% to over 90% in patients with either 1°-HPT or uremia. The specificity of this newly developed whole PTH(1-84) IRMA is the assurance, for the first time, of being able to measure only the biologically active whole PTH(1-84) without cross-reaction to the high concentrations of the aminoterminally truncated PTH fragments found in both normal subjects and patients. Because of the significant variations of pB% in patients, it is necessary to use the whole PTH assay to determine biologically active PTH levels clinically and, thus, to avoid overestimating the concentration of the true biologically active hormone. This new assay could provide a more meaningful standardization of future PTH measurements with improved accuracy in the clinical assessment of parathyroid function. (J Bone Miner Res 2001;16:605–614)

Key words: parathyroid hormone, immunoassay, hyperparathyroidism, uremia, parathyroid hormone fragment

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INTRODUCTION

The invention and evolution of immunoassays measuring human parathyroid hormone (PTH; parathyrin) has provided us with a better understanding of the biological and biochemical nature of this polypeptide hormone and a better tool for the clinical diagnosis and monitoring of the diseases related to primary hyperparathyroidism (1°-HPT), secondary hyperparathyroidism (2°-HPT), and hypoparathyroidism.[1–5] Circulating PTH is immunochemically heterogeneous and the midregional/C-terminal PTH fragments are known to be significantly accumulated in some disease conditions, for example, chronic renal failure.[6] Prior competitive immunoassays for PTH detect a mixture of different PTH fragments as well as the whole biologically active PTH(1-84); hence, these assays have not accurately assessed the level of circulating biologically active hormone and the function of the parathyroid glands. Because the whole or complete molecule of PTH(1-84) is the major circulating form of the serum biologically active hormone, which is capable of binding and activating the PTH-1 receptor on kidney and bone, the primary goal of developing and using intact PTH sandwich assays was to measure biologically active PTH(1-84) exclusively.[3]

Since 1987, commercially available "intact" PTH assays have greatly increased assay sensitivity and simplified the assay procedures for PTH measurement. However, the clinical use of these intact PTH assays is still fraught with challenges. For example, intact PTH levels frequently overestimate the presence and severity of parathyroid-mediated osseous abnormalities in uremic patients.[7–9] In addition, interlaboratory discordances of PTH values arose when different intact PTH kits from different manufacturers were used. One of the explanations could be that different paired antibodies with different specificities are used to form the sandwich assay for intact PTH. Indeed, recent studies have revealed that there are circulating non-(1-84) PTH fragments that interfere significantly with intact PTH measurements obtained from commercial assays in uremic patients.[10,11] One of these studies using high-performance liquid chromatography (HPLC) and different intact PTH assays has found that more than 30% of total immunoreactive intact PTH is comprised of non-(1-84) PTH fragments in this group of patients. Therefore, those intact PTH assays are not truly intact specific and still measure a mixture of the biologically active whole PTH(1-84) and large PTH fragments that show similar hydrophobicity as synthetic PTH-(7-84).[10]

It is our opinion that an optimal immunoassay for PTH should measure only the clinically significant, biologically active form of PTH, which is capable of binding to the G protein–linked PTH receptors,[12,13] which initiates signal transductions in the intracellular biochemical process resulting in the regulation of calcium metabolism. In addition to its specificity,[14,15] this optimal PTH assay should be sensitive, to allow diagnosis of hyperparathyroidism[16,17]; easy to perform; and of high performance in assay characteristics. To meet these goals for assaying PTH, we developed a whole PTH(1-84) immunoradiometric assay (IRMA) using a PTH(39-84) region–specific polyclonal capture antibody and a PTH(1-4) highly specific polyclonal label antibody. With these antibodies, this assay is restricted to measure only the authentic whole PTH(1-84) without any cross-reaction with the high levels of non-(1-84) PTH fragments found in patient samples. Clinical studies have shown that this specific whole PTH(1-84) assay unexpectedly provides a unique tool for the diagnosis of patients with parathyroid diseases. In studies with this new whole PTH IRMA and HPLC fractionated clinical samples, we clearly show that previously described non-(1-84) PTH fragments are amino-terminally truncated polypeptides and these PTH fragments are significantly present not only in uremic patients but also in patients with 1°-HPT and normal persons. Moreover, we further show that the ratio of full-length PTH(1-84) to aminoterminally truncated PTH fragments is significantly variable from patient to patient with HPT.

MATERIALS AND METHODS

Chemicals and reagents

Most chemicals were of reagent grade and were purchased from Sigma (St. Louis, MO, USA). Synthetic PTH(1-84) was from Peninsula Laboratories, Inc. (Belmont, CA, USA). Synthetic peptides of PTH(7-84), PTH(44-68), PTH(53-84), and PTH(39-84) were purchased from Bachem (Torrance, CA, USA). [Tyr34]PTH(1-34)amide {PTH(1-34)}, [Tyr34]PTH(2-34)amide {PTH(2-34)}, [Tyr34]PTH(3-34)amide {PTH(3-34)}, [Tyr34]PTH(4-34)amide {PTH(4-34)}, [Tyr34]PTH-(5-34)amide {PTH(5-34)}, and [Tyr34]PTHrP(1-34)amide {PTHrP(1–34)} fragments were synthesized by the Massachusetts General Hospital Polymer Core Facility (Boston, MA, USA). Cyanogen bromide–activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). One liter of 0.01 M phosphate-buffered saline (PBS; pH 7.4) contained 0.23 g sodium dihydrogen phosphate, 1.2 g disodium hydrogen phosphate, and 8.5 g sodium chloride. One liter 0.1 M glycine hydrochloride buffer (pH 2.5) contained 8.76 g sodium chloride. Assay wash buffer was 0.01 M PBS (pH 7.4) with 0.01% Triton X-100. Nichols intact PTH IRMA kit was purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA, USA).

Standards and controls for the whole PTH IRMA were prepared by adding synthetic PTH(1-84) to a normal human serum that did not show any detectable PTH level with the intact PTH assay. The concentrations of the standard set were 0, 10, 16, 46, 165, 700, and 2300 pg/ml. All standards and controls were aliquoted, lyophilized, and stored at 2–8°C.

Goat anti-PTH(39-84) polyclonal antibody coated onto 5/16-in polystyrene beads (Hoover Precision Products, Sault Ste. Marie, MI, USA) were used as the solid phase. The antibody was prepared by affinity purification. Briefly, synthetic PTH(39-84) peptide was conjugated covalently to Sepharose 4B gel using the manufacturer's suggested procedures by mixing the gel with the peptide at room temperature for 16 h. The peptide-bound Sepharose 4B gel was transferred to a chromatography column and the packed column was washed and equilibrated with 0.01 M PBS. Goat anti-PTH(39-84) antiserum was loaded onto the column. Unbound protein and other matrix components were

washed away using 0.01 M PBS and the specific goat anti-PTH(39-84) polyclonal antibody was eluted with 0.1 M glycine hydrochloride buffer. The eluted polyclonal antibody was neutralized and stored at 2–8°C. The purified goat anti-PTH(39-84) polyclonal antibody was attached physically onto the surface of the polystyrene beads by means of passive absorption.[5,18] The beads were blocked by Scancoat (Scantibodies Laboratory, Santee, CA, USA) and finally dried at room temperature. These antibody-coated beads were then stored at 2–8°C and were ready for assay use.

^{125}I-PTH(1-4) region–specific polyclonal antibody was used as the assay signal antibody. This antibody also was affinity-purified by the same procedure as described previously. The chloramine T method was used for the iodination of this most N-terminal PTH–specific antibody. A PD-10 column was used for the separation of the ^{125}I-labeled antibody from the free iodine. Selected fractions of labeled antibody were pooled and diluted using 0.01 M sodium phosphate–based buffer approximately to 300,000 disintegrations per minute (dpm) per 100 μl. This solution was the final tracer to be used in the whole PTH IRMA.

IRMA for whole PTH(1-84)

A single incubation step IRMA specific for the whole PTH(1-84) was developed and optimized with the previously mentioned assay reagents. Briefly, 200 μl of assay standards, controls, and patient samples were pipetted into appropriately labeled 12 mm × 75 mm polypropylene test tubes. One hundred microliters of ^{125}I-labeled PTH(1-4)–specific antibody tracer solution and one goat anti-PTH(39-84) polyclonal antibody–coated bead were added to all test tubes. The immunochemical reaction was conducted at room temperature with shaking at 170 rpm for 18–22 h. During this assay incubation period, the immunochemical reaction forming the sandwich of {solid-phase goat anti-PTH(39-84) antibody}–{whole PTH(1-84)}–{^{125}I-goat anti-PTH(1-4) antibody} takes place in correlation with the amount or concentration of whole PTH(1-84) in the test sample. All beads in the test tubes except the total count tube were washed with the wash solution, and the radioactive signals from each bead were counted for 1 minute using a gamma scintillation counter (ISO-Data, Palatine, IL, USA). The data were processed and calculated using nonlinear regression data reduction software.

Chromatographic separations

Sep-Pak Plus C_{18} cartridges (Waters Chromatographic Division, Milford, MA, USA) were used for the extraction of PTH from serum samples derived from single individuals or pools from up to 10 individuals among uremic patients, 1°-HPT patients, and normal persons. One cartridge was used for each 3 ml of serum and extracted volumes varied between 12 and 25 ml depending on the PTH concentration.[19] The eluted samples from the cartridges were first evaporated with nitrogen and then the residual volume was freeze-dried. All extracted samples were then reconstituted with 2 ml of 0.1% trifluoroacetic acid and chromatographed on a C_{18} μ-Bondapak analytical column (3.8 × 200 mm; Waters Chromatographic Division) using a noncontinuous linear gradient of acetonitrile (15–50% in 1.0 g/liter trifluoroacetic acid). After evaporation and freeze-drying, each 1.5-ml fraction was reconstituted to 1 ml with 0.7% bovine serum albumin (BSA) in H_2O. Both the whole PTH IRMA and the Nichols PTH IRMA were used to determine the PTH values in each fractionated sample. The recovery of intact PTH throughout all these procedures was 109 ± 10% in normal individuals, 70 ± 14% in renal failure patients, and 108 ± 4% in 1°-HPT.

Samples

One hundred and thirty-five normal human EDTA-plasma and serum samples were obtained from healthy laboratory staff members or donors, with an age ranging from 20 to 62 years (mean ± SD: 42 ± 12.6 years). Three hundred and eighteen patient samples of EDTA plasma (frozen/thawed once) were obtained from uremic patients with ongoing dialysis. The serum samples were collected and allowed to clot for approximately 30–40 minutes at room temperature and then centrifuged at 4°C. EDTA-plasma blood was collected into EDTA sample collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and immediately centrifuged at 4°C. The separated EDTA plasma and serum samples were stored at −20°C until used. One hundred and sixty-five samples (111 serum and 54 EDTA-plasma) from patients with surgically proven 1°-HPT were obtained from −70°C sample banks.

A stability study of whole PTH(1-84) in clinical samples was conducted with EDTA plasma, heparinized plasma, and serum. All three types of samples were drawn from three blood donors at the same time. One of the individuals was a patient with 1°-HPT, the other two were normal persons. Samples from only one of the normal persons, who had an original whole PTH(1-84) value of 9 pg/ml, were spiked with synthetic PTH(1-84) to an approximate level of 100 pg/ml. For this study the serum was obtained after routine blood clotting at room temperature for 30 minutes and centrifuged at 2–8°C for 10 minutes; for both EDTA-plasma and heparinized plasma the whole blood was placed immediately into an ice bath and centrifuged at 4°C. All samples were pooled, aliquoted at a 2-ml quantity, and incubated in 2-ml quantities at both room temperature and 2–8°C for 0–72 h, and frozen at −20°C until measured.

RESULTS

Performance characteristics of the whole PTH IRMA

Calibration curve and precision: An IRMA for whole PTH(1-84) was developed and optimized using the assay procedure described previously. A typical whole PTH IRMA standard curve is shown in Fig. 1. The affinity-purified antibodies used in the assay, either as capture antibody or as ^{125}I-labeled antibody, ensured the strong immunoreaction of antigen-antibody binding and low background of 526 ± 86 cpm (mean ± SD) for six iodinations. The intra-/interassay precision was determined by assaying

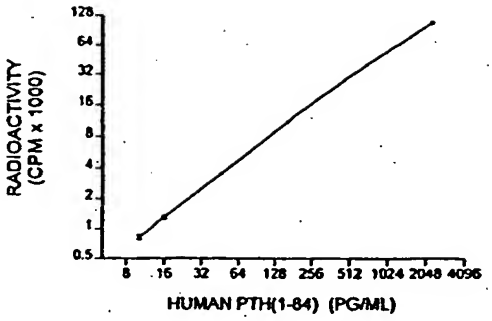

FIG. 1. A typical calibration curve obtained with the IRMA for whole PTH(1-84) as described in the Materials and Methods section. Data are expressed as means ± SD of triplicate measurements and are represented directly by the radioactivity (cpm × 1000).

two control samples with whole PTH(1-84) concentrations of 32 pg/ml and 340 pg/ml either by performing 60 replicate measurements in the same assay or in 40 different assays. The within-run variation was 6.1% and 2.3% and the between-run variation was 8.9% and 2.9%. No high-dose "hook" effect was observed after the addition to test samples of synthetic PTH(1-84) up to 20,000 pg/ml.

Analytical sensitivity: The assay detection limit was determined to be 1.0 pg/ml, which was the lowest measurable concentration of PTH value distinguishable from zero. It was determined by measuring the assay standard zero 22 times in the same assay and the value corresponding to the counts of 2 times of SD above the mean of the zero standard. This assay sensitivity was confirmed by validating with three independent production batches of the whole PTH reagents.

Linearity and analytical recovery: Three patient serum samples with PTH concentrations over 60 pg/ml were diluted 1:2, 1:4, and 1:8 with the assay zero standard. The percent recovery was determined after measurement of the diluted samples. Satisfactory assay linear recoveries of 93–112% were observed within the assay measurement range of 1.0–2300 pg/ml, respectively. Sample spiking recovery was determined by adding two different amounts of PTH into three patient serum samples with known whole PTH(1-84) values. The percentage of sample spike recovery was calculated following the assay of the spiked samples in comparison with the expected value. Recoveries from 99.3 to 113% were observed.

Analytical specificity and interference: Assay specificity to synthetic PTH(7-84) was studied by comparing this whole PTH IRMA with the Nichols intact PTH IRMA. Nearly 100% cross-reaction to this fragment was observed with the Nichols intact PTH assay, but no cross-reaction was detected with this newly developed whole PTH IRMA even at a PTH(7-84) concentration of 10,000 pg/ml (Fig. 2). The whole PTH IRMA also showed no cross-reaction to other PTH fragments, such as PTH(1-34), PTH(39-84), PTH(44-66), and PTH(53-84).

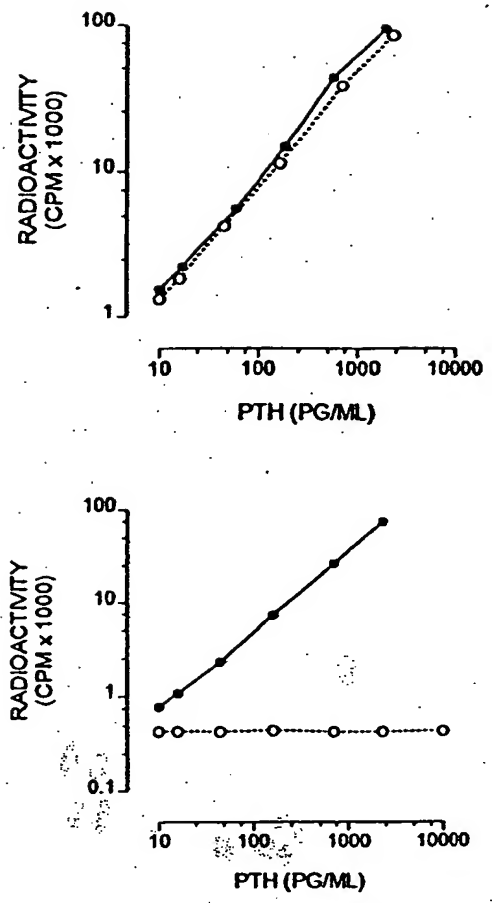

FIG. 2. Characterization of assay specificity for two PTH IRMAs [top, Nichols intact PTH IRMA; bottom, whole PTH IRMA; solid-circle, PTH(1-84); open-circle, PTH(7-84)].

Evaluating the specificity of tracer antibodies

The specificities of the two ^{125}I-labeled antibodies from the Nichols intact PTH IRMA and this new whole PTH IRMA were compared. Calibrators with a constant PTH(1-84) concentration of approximately 440 pg/ml were determined by both assays with increasing amounts (from 0 to 100,000 pg/ml) of coincubated aminoterminal PTH analogues. In the Nichols intact PTH IRMA, specific binding of ^{125}I-labeled tracer antibody to PTH(1-84) was reduced progressively by increasing concentrations of PTH(1-34), PTH(2-34), PTH(3-34), PTH(4-34), and PTH(5-34). In the whole PTH IRMA, in contrast, the bound signal of ^{125}I-labeled antibody was only competitively inhibited by PTH(1-34). No binding reduction could be determined by increasing concentrations of PTH(2-34), PTH(3-34), PTH(4-34), and PTH(5-34) (Fig. 3). Increasing concentrations of PTHrP(1-34) had no inhibitory effect on the ^{125}I-labeled antibodies in both assays.

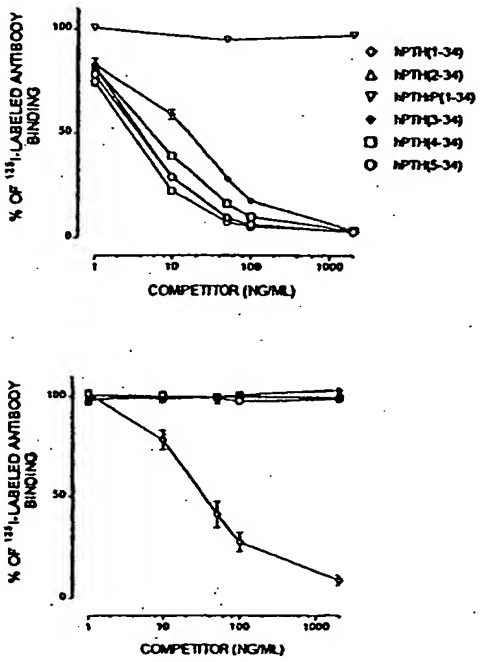

FIG. 3. Characterization of two tracer antibodies used in the Nichols intact PTH IRMA (top) and the whole PTH IRMA (bottom). Data are expressed as means ± SD of duplicate measurements and are represented by percentage changes from the original uninhibited antibody binding.

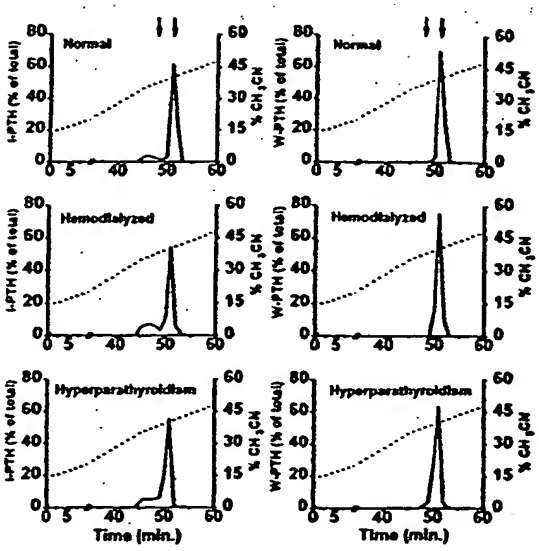

FIG. 4. HPLC profiles of immunoreactive PTH present in serum of a normal individual, a 1°-HPT patient, and a hemodialysis patient. Profiles were analyzed using the Nichols intact PTH IRMA and the whole PTH IRMA. Results are expressed as a percentage of the total immunoreactivity. A peak distinct from PTH(1-84) is detected by the intact PTH assay but not by the whole PTH(1-84) assay.

Assay validations using chromatographic fractionated samples

Figure 4 shows the two different immunoreactive PTH profiles with HPLC fractionated samples from 1 normal person, 1 patient with 1°-HPT, and one patient with 2°-HPT caused by chronic renal failure. The elution position of PTH(1-84) and of PTH(7-84), a prototype of those circulating non-(1-84) PTH fragments, also is indicated. Two immunoreactive peaks were detected in samples from all three groups using the Nichols intact PTH IRMA; the first peak corresponded to the aminoterminal truncated PTH with similar hydrophobicity and elution position as PTH(7-84) and the second one to the immunoreactive PTH(1-84), whereas, only one major immunoreactive peak corresponding to the elution position of PTH(1-84) was detected in all three samples using the newly developed whole PTH IRMA. Results of all HPLC runs are summarized in Table 1. There was a good agreement between the results of whole/intact PTH ratio and the amount of PTH(1-84) obtained by planimetric evaluation of the intact PTH HPLC profiles in the populations studied.

Sample stability for the whole PTH(1-84) measurement

The stability of whole PTH(1-84) was studied as follows: (1) in serum, EDTA plasma, and heparinized plasma; and (2) at 2–8°C and at room temperature (RT). The results indicated that: (a) whole PTH(1-84) in EDTA plasma and heparinized plasma is stable (<5% degradation) at 2–8°C or RT for at least 24 h; and (b) whole PTH(1-84) in serum, however, is only stable for 6 h at RT (>10% degradation) and for about 24 h at 2–8°C (Fig. 5). Additionally, a study of four times sample freeze/thaw showed that both serum and EDTA plasma were relatively stable with a <5% decrease in immunoreactivity.

Assay correlation and clinical evaluation

The normal range of whole PTH(1-84) was found to be 7–36 pg/ml (mean ± SD: 22.7 ± 7.2 pg/ml; n = 135) for EDTA plasma.

To study the correlation and difference between whole PTH(1-84) and conventional intact PTH levels in normal persons, 56 normal human EDTA plasma samples were measured at the same time with two different PTH assays, the newly developed whole PTH IRMA and the Nichols intact PTH IRMA. All the samples had measurable whole PTH(1-84) values. There were also measurable PTH values in all normal samples using the Nichols intact PTH IRMA. However, all intact PTH values measured by the Nichols PTH IRMA were higher than the whole PTH(1-84) values (Table 2) revealing an average of about 33% PTH fragments being comeasured with PTH(1-84) by intact PTH assay. Paired Student's t-test showed a significant difference ($p <$ 0.0001) between the two sets of PTH values with these two PTH IRMAs (Fig. 6, bottom). The correlation of these two

TABLE 1. COMPARISON OF HPLC PROFILE RESULTS WITH WHOLE/INTACT PTH RATIOS IN NORMAL INDIVIDUAL, RENAL FAILURE, AND 1°-HPT PATIENTS

						HPLC results		
						Intact PTH (%)		
Groups	Ca^{2+} (mmol/liter)	Creatinine (μmol/liter)	Whole PTH (pg/ml)	Intact PTH (pg/ml)	Whole/intact PTH	PTH(1-84)	PTH fragment	Recovery (%)
Normals (n = 5)	2.25 ± 0.07	85.2 ± 6.6	24.1 ± 7.5	30.2 ± 9.5	0.8 ± 0.00	85.3 ± 1.5	14.7 ± 1.5	109 ± 9.8
Renal Failure (n = 5)	2.47 ± 0.12	864 ± 82	226 ± 185	337 ± 280	0.7 ± 0.07	65.3 ± 1.9	34.7 ± 1.9	70.3 ± 14.3
1°-HPT (n = 3)	2.58 ± 1.1	84 ± 23	53.5 ± 48.1	68.7 ± 42.0	0.69 ± 0.24	70.1 ± 9.7	29.9 ± 9.7	107.9 ± 4.5

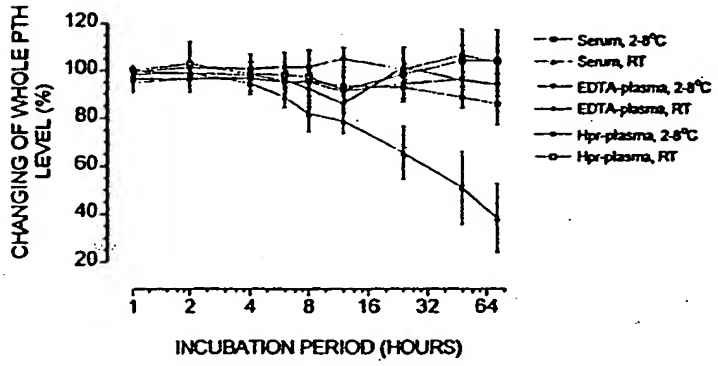

FIG. 5. Sample stability for the whole PTH(1-84) measurement. Data are expressed as means ± SD of duplicate measurements and are represented by percentage changes from the original concentrations.

groups of PTH values also was calculated (r = 0.923; slope = 1.456).

To ensure that this difference of PTH values was only caused by the specific antibody-antigen binding and not caused by differences in assay matrix or calibrators, different amounts of synthetic PTH(1-84) were spiked into several normal human sera with nondetectable PTH levels and measured with the previously mentioned two PTH assays. The result showed these two assays detect PTH(1-84) equally (r = 0.999; slope = 1.04; Fig. 6, top).

Human PTH values from a sample group of 318 uremic patients with ongoing hemodialysis also were determined with these two assays. The results showed that the PTH values displayed a heterogeneous distribution pattern in normal, below-normal, and elevated levels using both assays. The mean and median for the whole PTH(1-84) in this group also differed significantly from that obtained with the Nichols intact PTH assay ($p < 0.0001$; paired Student's t-test; Table 2). Figure 7 shows the correlation comparison of these two assays in the uremic patient group (r = 0.977; slope = 1.482). Samples from 165 patients with surgically confirmed 1°-HPT with parathyroid adenomas also were measured using the whole PTH IRMA (mean ± SD: 116.7 ± 129.6 pg/ml) and the Nichols intact PTH IRMA (mean ± SD: 200.3 ± 208.9 pg/ml). An effective differentiation of this patient group from normal persons was observed (Fig. 8). The overall clinical diagnostic sensitivity with a single sample PTH measurement was 93.9% (155/165) using whole PTH IRMA and 91.5% (151/165) using Nichols intact PTH IRMA.

The ratios of whole PTH to intact PTH or percentage of biologically active PTH(1-84) (pB%) to the total immunoreactive intact PTH were calculated for all 318 uremic patients and 165 1°-HPT patients. The results display an almost Gaussian distribution pattern from 20% to >90% in both patient groups (Fig. 9). This inconsistent pB% may be the result of variations in peripheral clearance of PTH or the glandular secretion of PTH(1-84) and its fragments.[20] This finding further indicates that currently available intact PTH values could not assess accurately the parathyroid function of patients.

DISCUSSION

The present report describes for the first time an immunoassay that measures only the biologically active whole PTH(1-84) without any cross-reactivity to PTH fragments, although current intact PTH immunoassays have been used and presumed to be specific for intact PTH for over 10 years. One study evaluated serum intact PTH levels in conjunction with histological analyses of iliac crest bone biopsy specimens.[7] It was found that serum intact PTH assays overestimate the presence and severity of PTH-

TABLE 2. COMPARISON OF INTACT PTH VALUES, WHOLE PTH(1-84) VALUES, AND pB% {(WHOLE PTH VALUE/INTACT PTH VALUE) × 100%} IN PATIENTS WITH UREMIA AND SURGICALLY PROVEN PRIMARY HPT WITH NORMAL PERSONS

	Uremic patients (n = 318)			*1°-HPT patients (n = 165)*			*Healthy controls (n = 56)*		
	Intact hPTH (pg/ml) [Nichols]	*Whole hPTH (pg/ml) [Scantibodies]*	*pB%** (%)	*Intact hPTH (pg/ml) [Nichols]*	*Whole hPTH (pg/ml) [Scantibodies]*	*pB%†* (%)	*Intact hPTH (pg/ml) [Nichols]*	*Whole hPTH (pg/ml) [Scantibodies]*	*pB%* (%)
Minimum	3.4	1.1	15	32	23	22	13	8	35
Maximum	5230	1388	99	2000	909	98	67	37	88
Mean	442	254	55	200	117	59	41	26	65
Median	300	154	54	143	75	58	35	22	67
SD	515	223	15	209	130	16	25	16	16

* $p < 0.001$ for the ratio of whole/intact hPTH between uremic patients and healthy controls (two-tailed Mann-Whitney test).
† $p < 0.01$ for the ratio of whole/intact hPTH between 1°-HPT patients and healthy controls (two-tailed Mann-Whitney test).

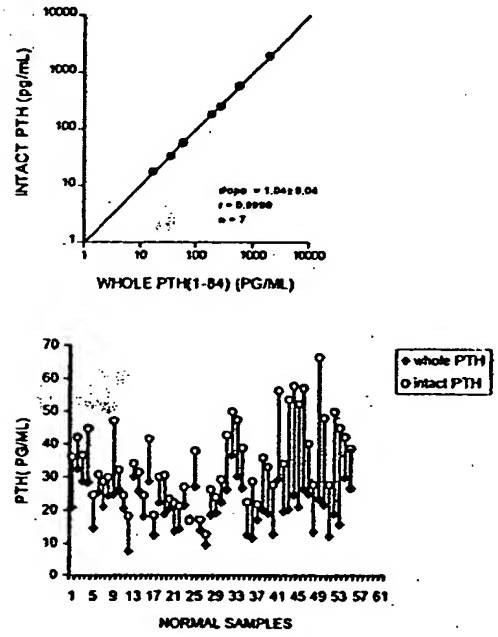

FIG. 6. Assay correlation studies of 56 normal persons (bottom; open circle, Nichols intact PTH IRMA; solid diamond, whole PTH IRMA) and from 7 artificial samples containing only whole PTH(1-84) (top). Data are expressed as means of duplicate measurements.

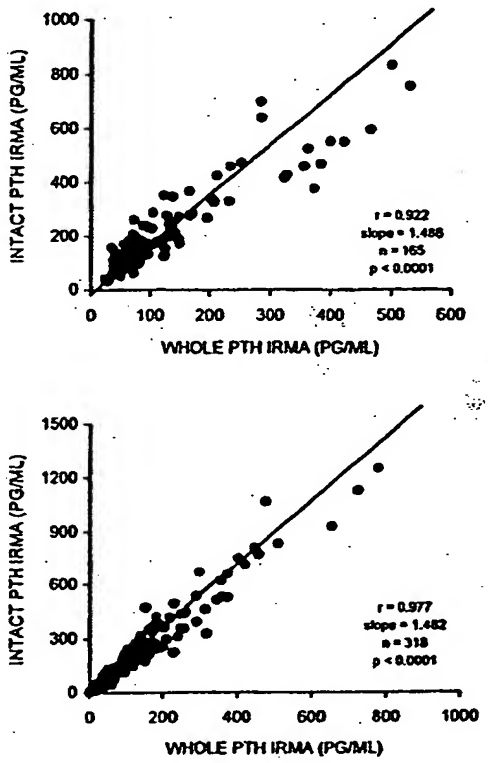

FIG. 7. Assay correlation study of 165 1°-HPT samples (top) and 318 uremic samples (bottom) using the Nichols intact PTH IRMA and the whole PTH IRMA. Paired Student's *t*-test was used for *p* value calculation.

mediated osseous abnormalities associated with uremia. Although at that time the reason for this overestimation was not elucidated, it might have been explained partially by this work in combination with recent studies.[10,11,21] It has been shown that the commercially available intact PTH assays measure both PTH(1-84) and non-PTH(1-84) fragments that are present in significant concentrations in the blood of uremic patients.[10] Therefore, these intact PTH assays are not truly intact PTH specific and the term "intact" is used inaccurately.

The specificity studies of the tracer antibody show that the newly developed anti-PTH(1-4) antibody is truly amino-terminal PTH specific. In fact, it is directed at the first amino acid of the aminoterminal polypeptide (Fig. 3), therefore, being able to bind to PTH(1-34) but not PTH(2-34), -(3-34), -(4-34), and -(5-34). By contrast, the tracer anti-

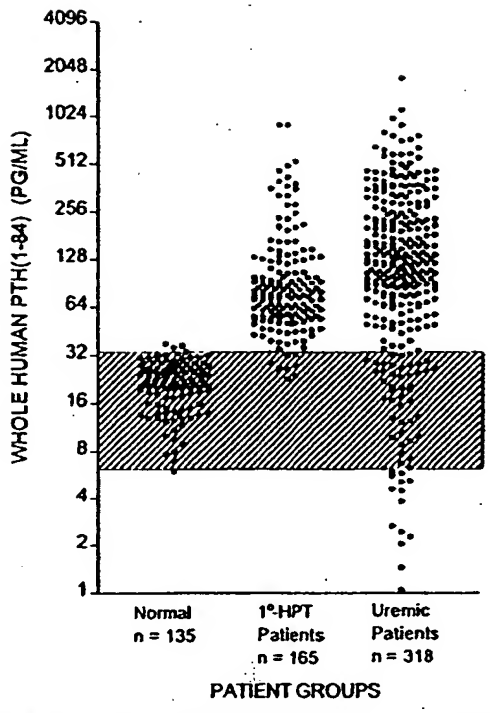

FIG. 8. Scatterplot of whole PTH(1-84) values in healthy controls and various patient groups. Shaded area indicates the plasma normal range (7–36 pg/ml) of whole PTH(1-84). The y axis is expressed by $\log_2$ scale. The whole PTH levels of 10 1°-HPT patients were located in the upper normal range and the overall diagnostic sensitivity was 93.9% (155/165).

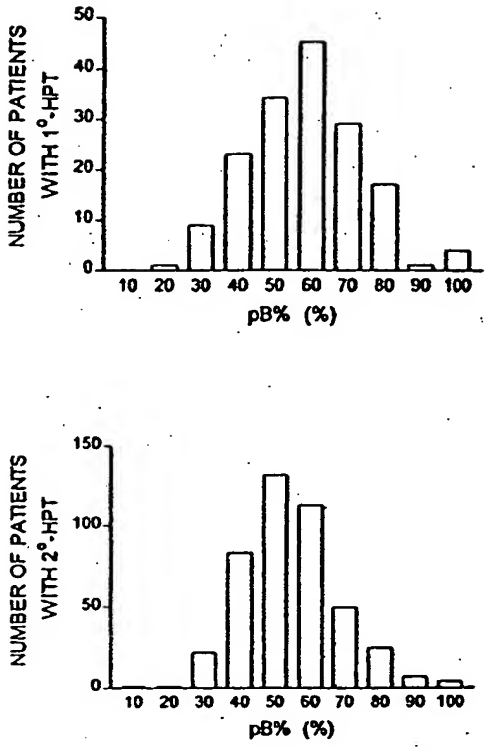

FIG. 9. Histogram showing the frequency distribution of the pB% in the pool of the total immunoreactive intact PTH value in patients with 1°-HPT (n = 165, top) and 2°-HPT (n = 318, bottom) of uremia.

body used in Nichols intact PTH IRMA is broadly PTH(1-34) specific and, therefore, cross-reacts with PTH(2-34), -(3-34), -(4-34), and -(5-34). It is the specificity of the tracer antibody used in this new whole PTH assay that ensures that this unique assay only detects the full-length PTH(1-84) without cross-reaction to any aminoterminally truncated PTH fragments. In theory, this assay also could detect carboxy-terminally slightly truncated PTH fragments, which should be the same for other intact PTH assays. Using the commercially available synthetic aminoterminally truncated PTH fragment, PTH(7-84) other intact PTH assays (Incstar, Diagnostic System Laboratory, Diagnostic Product Corp.) show variable cross-reactivity of 60–80% from assay to assay.[22] The whole PTH IRMA was thoroughly designed and developed in a coated bead format and single incubation step. It is easy to perform and presents a clinically adequate measurement range of 1–2300 pg/ml with acceptable assay performance characteristics, including linearity, sample spiking recovery, and intra-/interassay precision.

The study of the chromatographically fractionated serum samples from normal population and patients with either 1°-HPT or 2°-HPT further shows that there are two forms of PTH or immunoreactive peaks detected by the Nichols intact PTH IRMA. The first immunoreactive peak corresponds to non-PTH(1-84) fragments migrating on HPLC to a similar position as PTH(7-84) and the second peak corresponds to the full-length PTH(1-84).[10] However, when the same samples were measured with the whole PTH IRMA, only one immunoreactive peak was detected corresponding to the full-length PTH(1-84). Comparing the specificity of the antibodies used in these two assays, it is quite obvious that the non-PTH(1-84) corresponds to aminoterminally truncated PTH fragments. Moreover, these HPLC fractionated patient sample measurements further show that these aminoterminally truncated polypeptides are present in significant amounts not only in uremic patients, but also in the normal population and in patients with 1°-HPT (Fig. 4; Table 1). The exact molecular structure of these PTH fragments should be further determined by isolating and analyzing their amino acid sequences using pools of patient serum samples.

The correlation study of whole PTH IRMA to Nichols intact PTH IRMA from samples that contain only synthetic PTH(1-84) indicates that the two assays are nearly equivalent in their detection of PTH(1-84) (Fig. 6, top). However, when clinical samples from a normal population group and patients with 1°-HPT or 2°-HPT were used for the study, significant differences with higher intact than whole in the

absolute PTH values were found ($p < 0.0001$, paired t-test) in all three groups (Figs. 6 and 7; Table 2).

The clinical significance of this newly developed whole PTH IRMA was shown in three population groups. The normal range of whole PTH(1-84) was 7–36 pg/ml for samples of EDTA plasma. Samples of EDTA plasma are preferred for whole PTH measurement because the hormone appears to be more stable in EDTA plasma than in the serum (Fig. 5). There is an unexpected distinction in whole PTH(1-84) levels of patients with 1°-HPT from the normal population with an overall diagnostic sensitivity of 93.9% ($n = 165$) in this study. A diagnostic sensitivity of 91% also was found with Nichols intact PTH IRMA in this study. However, Kao et al.[23] evaluated 361 patients with surgically proven 1°-HPT in whom intact PTH had been determined with an immunochemiluminometric assay and found 45 patients to have an intact PTH value below the upper limit of normal. Endres et al.[24] also reported that only 21 of 29 cases of 1°-HPT had values above the normal level when the Nichols Allégro intact PTH assay was used. These early studies indicated a diagnostic sensitivity of intact PTH assay of about 72.4–87.5% only. Most recently, Silverberg et al.[25] reported a prospective clinical validation using whole PTH assay, Nichols intact PTH assay, and a midregional PTH competitive assay. In her study, a well-defined group of patients with mild 1°-HPT was chosen and the clinical diagnostic sensitivities were 96% for whole PTH assay, 76% for intact PTH assay, and 54% for a midregional PTH assay. Significant statistical differences were found between each assay in this study. Whole PTH(1-84) values from 318 uremic patients displayed a heterogeneous distribution pattern with both normal and elevated levels.

This study has shown that there is no consistent percentage of aminoterminally truncated PTH fragments (Fig. 9; Table 2). It is inconsistent percentage of aminoterminally truncated PTH fragments among patients with HPT that could easily give rise to two previously unforeseen major problems in the clinical decisions based on available intact PTH assays for evaluating the function of the parathyroid glands. First, because most intact PTH assays have >60% cross-reaction[10] to the PTH fragments and the ratio of whole PTH/intact PTH or the pB% is not consistent even in patients in the same disease condition, the parathyroid function will always be overestimated and inconsistently estimated in different degrees by intact PTH assays measuring both the full-length whole PTH(1-84) and its aminoterminally truncated fragments. Second, because of the significantly different molar rates of cross-reactivity of commercially available intact PTH assays, interlaboratory discordance of PTH levels have been observed from the use of different intact PTH assays. Theoretically, the aminoterminally truncated PTH fragment is a naturally produced polypeptide, which is able to bind to PTH/PTH-related protein (PTHrP) receptors. One preliminary in vivo study with parathyroidectomized rats showed an 80% decreased calcemic response for a 1:1 molar ratio of infused PTH(7-84) and PTH(1-84) compared with PTH(1-84) alone.[26] The biological importance of these aminoterminally truncated fragments that have been shown to act as PTH antagonist or inhibitor appears to regulate eventually the sensitivity of PTH/PTHrP receptors and warrants further investigation. These PTH fragments also could be ligands for a thus far unisolated receptor for the carboxy-terminal part of PTH. However, whether this receptor plays a role in the regulation of calcium metabolism is not known.[27]

In summary, a novel IRMA was developed that only detects biologically active whole PTH(1-84) without cross-reaction to the aminoterminally truncated PTH fragments. The assay uses only a single incubation procedure. The PTH(1-84) specificity of the new assay was defined by tracer antibody evaluation, cross-reactivity experiments, and measurements of HPLC fractionated patient samples. With this whole PTH IRMA, we first showed that previously described non-(1-84) PTH fragments[10] should be aminoterminally truncated. The presence of these aminoterminally truncated PTH fragments was shown not only in uremic patients, but also in 1°-HPT patients and normal persons. Moreover, the percentage concentration of the biologically active whole PTH(1-84) in the pool of total immunoreactive intact PTH is significantly variable from patient to patient, even in patients with the same type of HPT and, thus, it is impossible to interpret biologically active PTH levels with current intact PTH assays. The new whole PTH IRMA is clinically significant in differentiating patients with 1°-HPT and 2°-HPT from the normal population in measuring PTH(1-84) exclusively. Because of the immunological heterogeneity of circulating PTH, this new assay model could be applied as a more meaningful and standardized method for the measurement of biologically active and hence clinically significant PTH.

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